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# MODULATION OF HAEM OXYGENASE-1 BY PHYTOCHEMICALS: A NOVEL STRATAGEM IN THE MITIGATION OF CARDIOVASCULAR TISSUE DYSFUNCTION

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# ABSTRACT

***Background:*** The haem oxygenase-1(HO-1) pathway plays a key role in the preservation of tissue integrity against damaging insults such as oxidative stress and inflammatory injuries. Thus, HO-1 is regarded as a potential therapeutic target in conditions which are mediated by oxidative stress and inflammation, e. g ischaemic heart disease, septic shock, and ischaemia reperfusion injury.

***Aims:*** The objective of the study was to investigate if the up-regulation of the HO-1 expression by two plant-derived polyphenolic compounds, i.e. curcumin and 2'-hydroxychalcone (2-HC) offers protection to against stress-induced cellular damage in cardiovascular tissue. And to elucidate the molecular and cellular signalling mechanisms involved.

***Methods:*** Parallel experiments were conducted using **1)** Lipopolysaccharide (LPS)-induced model of inflammation in RAW 264.7 murine macrophages, the inflammatory response was assessed using nitrite assay, Western blot for the determination of the inducible nitric oxide synthase (iNOS), HO-1 protein expression and the activation of the transcriptional factor NF-KB. The role of HO-1 in this process was elucidated using small interfering RNA (siRNA). The involvement of the mitogen-activated protein kinase (MAPK), the phosphoinositide 3-kinase pathway (PI3K) pathway and the nuclear transcription factor (Nrf2) was also studied. ***2)*** We also utilised Carbon Monoxide-Releasing Molecules (CO-RMs) as a method to study the effects of CO, one of the products of HO-1 enzymatic activity, *in vitro* model of inflammation. ***3)*** *In vitro* model of $H_2O_2$-induced oxidative stress, using human cardiac myoblasts. ***4)*** *In vitro* model of ischaemia-reperfusion injury and cold

preservation in cardiac myoblasts. HO-1 mRNA expression during hypothermia was determined using RT-PCR.

***Results:*** The major findings indicated that up-regulation of HO-1 gene and protein expression resulted in the protection of cells against oxidative and inflammatory injuries. We also found that 2-HC is a potent inducer of HO-1 protein expression in bovine aortic endothelial cells and in macrophages, in mechanisms which involve the activation of PI3K pathway. Treatment of macrohages with 2-HC resulted in the reduction of pro-inflammatory cytokines (TNF-α), and down regulation of iNOS expression but did not inhibit the activation of NF-KB. Using HO-1 siRNA, we confirmed that HO-1 mediated the anti-inflammatory actions of 2-HC. We also identified two novel CO-RMs (CORM-43 and CORM-319) as potent anti-infallamtory compounds. We also found that curcumin mitigated the cellular dysfunction-mediated by $H_2O_2$. This study also demonstrated that cold storage of human cardiac myoblasts caused marked increase in cytotoxicity primarily due to loss of cell membrane integrity as a result of necrosis. The addition of curcumin to the cold preservation solution (Celsior solution) resulted in preservation of cellular membrane integrity as well as cellular metabolism in cells exposed to cold storage and rewarming.

***Conclusion:*** up-regulation of HO-1 is an effective therapeutic strategy to ameliorate cardiovascular injury and protect cardiac tissue during pathological conditions mediated by oxidative and inflammatory stresses.

# QUOTATION

*There are two kinds of truth; the truth that lights the way and the truth that warms the heart. The first of these is science, and the second is art*

*Raymond Chandler*

1888-1959

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# GLOSSARY OF ABBREVIATIONS

| | |
|---|---|
| **ARE** | Antioxidant responsive elements |
| **CO** | Carbon monoxide |
| **CO-RM** | Carbon Monoxide-Releasing Molecule |
| **GSH** | Reduced glutathione |
| **$H_2O_2$** | Hydrogen peroxide |
| **HO-1** | Haem oxygenase-1 |
| **HO-2** | Haem oxygenase-2 |
| **HO-3** | Haem oxygenase-3 |
| **HSP** | Heat shock protein |
| **I/R** | Ischaemia-reperfusion |
| **iNOS** | Inducible nitric oxide synthase |
| **LPS** | Lipopolysaccharide |
| **MAPK** | Mitogen-activated protein kinase |
| **NF-KB** | Nuclear factor kappa B |
| **NO** | Nitric oxide |
| **NOS** | Nitric oxide synthase |
| **PI3K** | Phosphoinositol 3 kinase |
| **ROS** | Reactive oxygen species |
| **SnPPIX** | Tin Protoporphyrin |
| **TNF-α** | Tumour necrosis factor-α |

# 1 INTRODUCTION

## 1.1 Anatomy and Physiology of the Cardiovascular System

The cardiovascular system consists of the blood, heart, and blood vessels. The heart is the pump that circulates the blood though blood vessels. It lies in the mediastinum, about two-thirds of its mass is to the left of the midline. The heart has four chambers, the two superior chambers are the left and right atria, and the two inferior chambers are the left and right ventricles (Figure 1.1 A). As each chamber of the heart contracts, it pushes a volume of blood into a ventricle or out of the heart into arteries. The right ventricle receives blood from the right atrium and then pumps it to the lungs through the pulmonary circulation. Oxygenated blood enters the left atrium through the pulmonary veins and then exists through the mitral valve to the left ventricle. The left ventricle then pumps it to the systemic circulation. There are four valves which open and close to ensure one way flow of blood by opening to let blood through and closing to prevent back flow (Figure 1.1 B). The blood supply of the heart comes from coronary arteries. The wall of the heart consists of three layers, the epicardium (the external layer), the myocardium (middle layer) and the endocardium (inner layer). The epicardium is the thin, transparent outer layer of the wall. It is composed of mesothelium and delicate connective tissue that imparts a slippery texture to the outermost surface of the heart. The myocardium is cardiac muscle tissue, making up the bulk of the heart and responsible for its pumping action. The endocardium is a thin layer of endothelium overlying a thin layer of connective tissue. Blood vessels form a closed system of tubes that carries blood away from the heart, transports it to the tissues, and then returns to the heart. Arteries are vessels that carry blood

from the heart to the tissues. Arterioles are small arteries that deliver blood to capillaries, which are microscopic blood vessels through which nutrients and oxygen are exchanged between blood and tissue. Blood then get transported through small vessels (venules) that merge to form veins, which drain blood into the heart.

Reviewed from : Anatomy and Physiology in Health and Illness, Janet S. Ross, Kathleen J.W. Wilson, Anne Waugh and Allison Grant (2001).

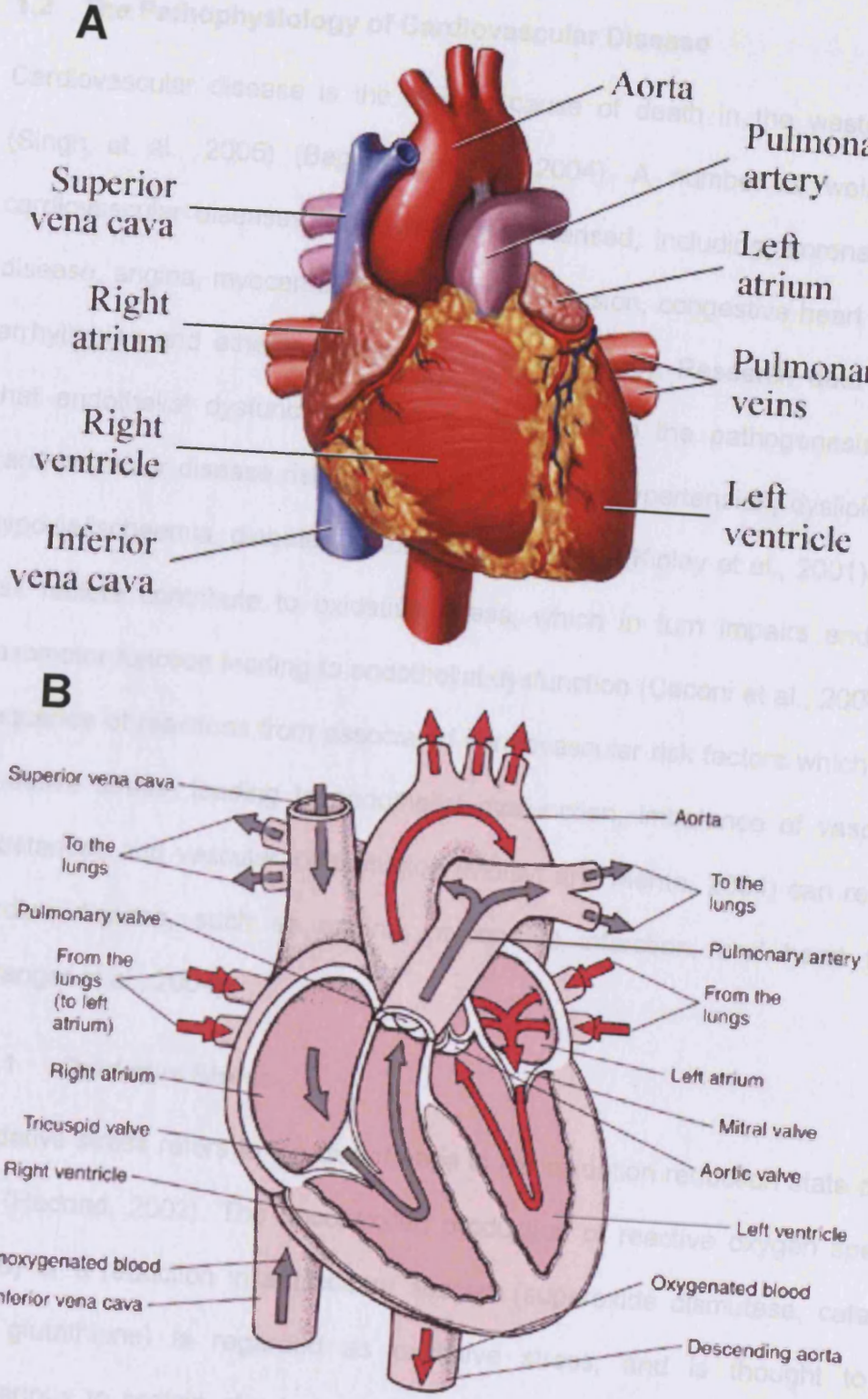



**Figure 1.1: A schematic representation of anatomy (A), and physiology (B) of the heart. Adapted from www.merck.com**

## 1.2 The Pathophysiology of Cardiovascular Disease

Cardiovascular disease is the leading cause of death in the western world (Singh et al., 2005) (Baguneid et al., 2004). A number of well defined cardiovascular diseases have been characterised, including, coronary artery disease, angina, myocardial infarction, hypertension, congestive heart disease, arrhythmias and atherosclerosis (Chen et al., 2000). Research data suggest that endothelial dysfunction plays a major role in the pathogenesis of the cardiovascular disease risk factors, which include hypertension, dyslipidaemia, hypoxia/ischaemia, diabetes, smoking, and obesity (Kinlay et al., 2001). These risk factors contribute to oxidative stress, which in turn impairs endothelial vasomotor function leading to endothelial dysfunction (Ceconi et al., 2003). The sequence of reactions from associated cardiovascular risk factors which cause oxidative stress, leading to endothelial dysfunction, imbalance of vasoactive substances and vascular inflammation (Molavi and Mehta, 2004) can result in cardiac disease, such as angina, myocardial infarction, and heart failure (Granger et al., 2004).

### 1.2.1 Oxidative Stress

Oxidative stress refers to the disturbance in the oxidation reduction state of the cell (Haddad, 2002). The uncontrolled production of reactive oxygen species (ROS) or a reduction in antioxidant species (superoxide dismutase, catalase and glutathione) is regarded as oxidative stress, and is thought to be deleterious to protein structure and function (Halliwell and Cross, 1994). An increasing body of evidence suggests that oxidative stress is involved in the pathogenesis of cardiovascular diseases (Ceconi et al., 2003). The important forms of ROS in the cardiovascular system include superoxide ($O_2^-$), hydrogen

peroxide ($H_2O_2$), hydroxyl radical ($^{\cdot}OH$) and nitric oxide (NO) (Parthasarathy et al., 2001). Oxygen is an abundant molecule in biological systems, and it undergoes reduction to form superoxide ($O_2^-$) (Figure 1.3) , by enzymes such as xanthine oxidases (Chen et al., 2000). The bulk of oxygen reduction in most cells, such as in the heart, occurs by the mitochondrial cytochrome oxidase pathway (Molavi and Mehta, 2004), these radicals are capable of destroying biomolecules through oxidation (Ceaser et al., 2004) (Figure 1.3). However, in a physiologic environment, various ROS are formed by different systems, whereby they exert physiologic actions; for example, oxygen radicals are key intermediates in metabolic reactions (Halliwell and Cross, 1994). Furthermore, in the vascular endothelium, NO radicals cause relaxation of vascular smooth muscle (Singh et al., 2005). In addition, phagocytic cells synthesize hypochlorous acid through oxidation of chloride ions by $H_2O_2$ through a reaction catalyzed by myeloperoxidase (Singh et al., 2005). However, excessive ROS production causes lipid peroxidation and depletion of cellular energy via disruption of mitochondrial enzymes and nucleic acids (Holtzclaw et al., 2004), which lead to functional damage of the endothelium (Hamilton et al., 2004).

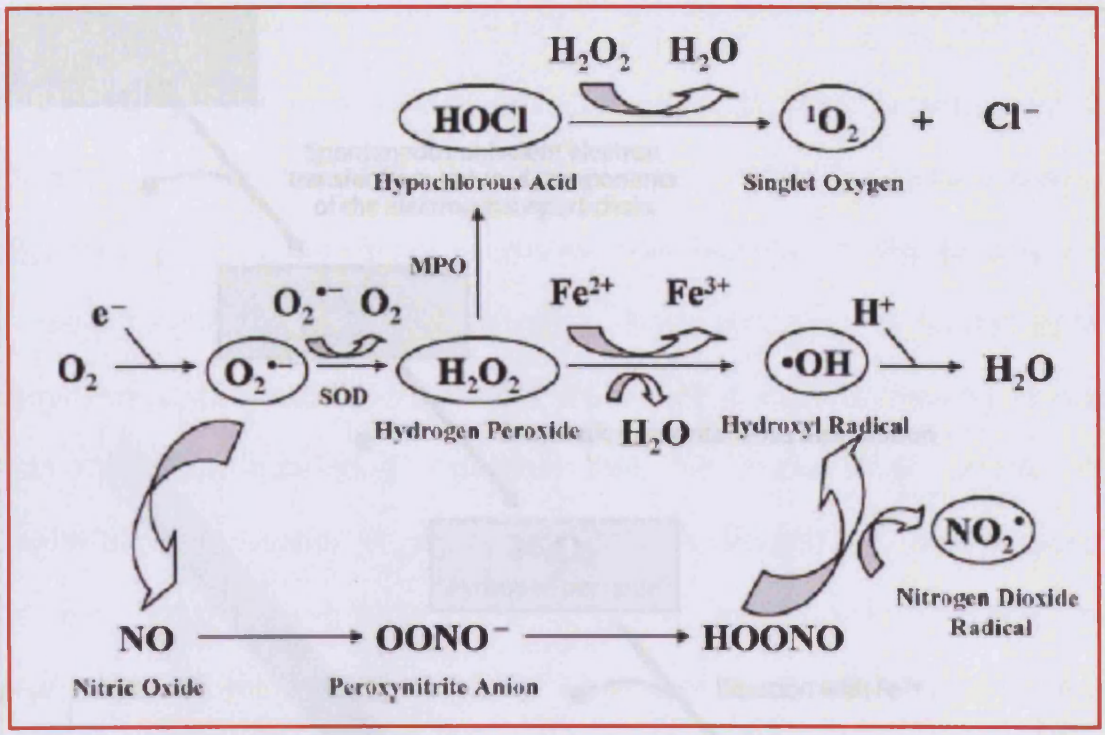



**Figure 1.2: A schematic model of the pathways leading to the generation of ROS/RNS**

Superoxide ($O_2^-$) anion is metabolized via the dismutation reaction $2O_2^{\cdot} + 2H \rightarrow O_2 + H_2O_2$, which is catalyzed by superoxide dismutase (SOD), a cytoplasmic enzyme that is constitutively expressed, and a mitochondrial enzyme that is induced in response to oxidant stress. The $H_2O_2$ produced by the dismutation of $O_2^{\cdot}$ is converted by one pathway to $H_2O$ and $O_2$ by catalase (CAT) in peroxisomes and by glutathione peroxidase in the cytoplasm, at the expense of reduced glutathione (GSH), leading to the formation of oxidized glutathione disulphide (GSSG) that is recycled back to GSH by glutathione reductase (GSSG-RD). $H_2O_2$ could be further converted by another pathway involving iron into hydroxyl radical (OH), an injurious ROS causing cellular damage. This iron-catalyzed reaction, known as the Fenton-like reaction, is impeded by the iron chelator desferrioxamine (DSF), which is also capable of neutralizing the toxicity of OH. Phagocytic cells synthesize hypochlorous acid through oxidation of chloride ions by $H_2O_2$ through a reaction catalyzed by myeloperoxidase. NO reacts rapidly with superoxide anion (O.) leading to the formation of $ONOO_2$, which reacts with H+ to form ONOOH potent oxidant species that has been shown to cause nitration of proteins, as well as lipid peroxidation and cytotoxicity (Motterlini et al., 2002b).

Adapted from reference (Haddad, 2002).

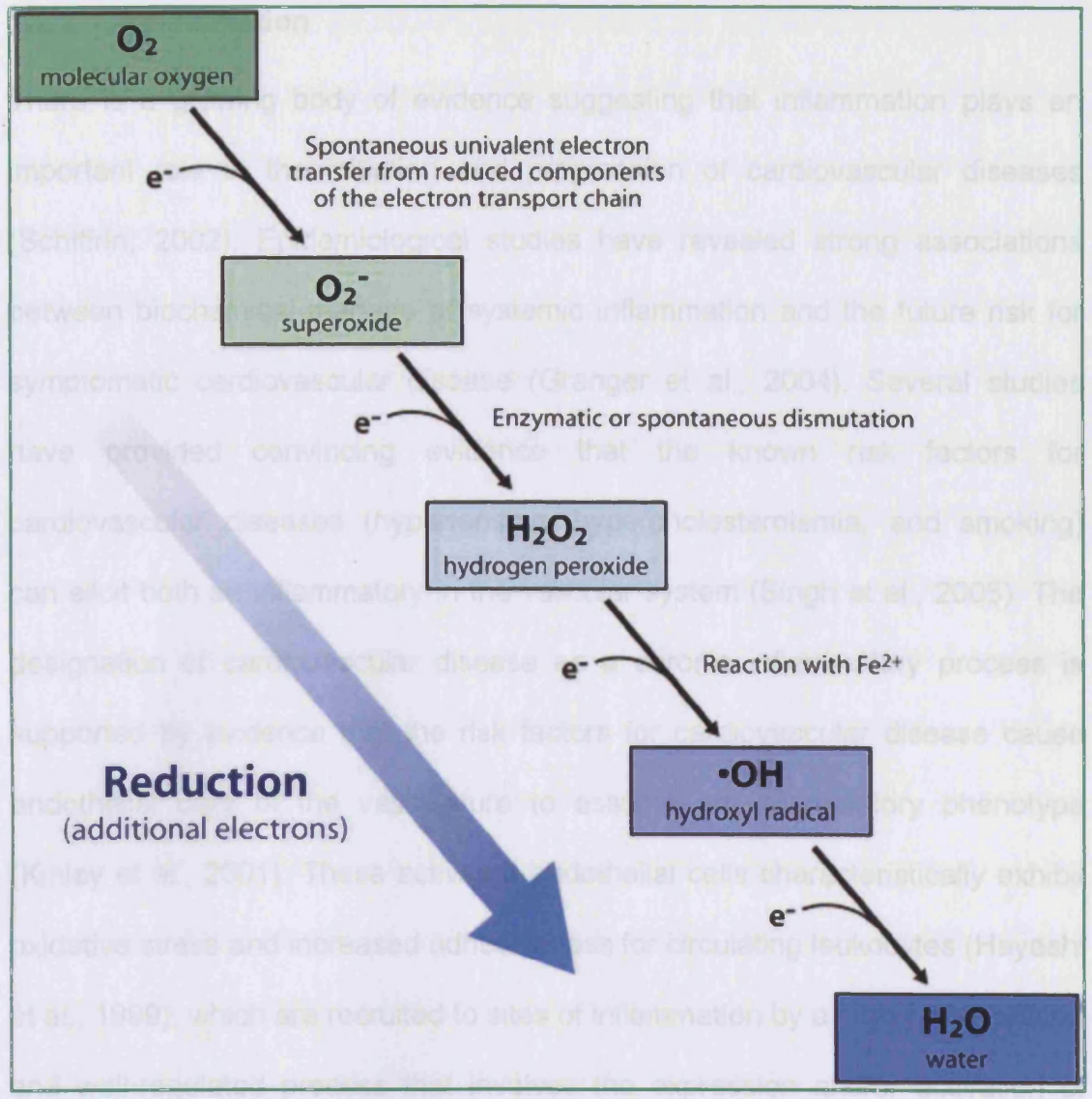



**Figure 1.3: Selective dismutation of ROS**

Superoxide ($O_2^-$) anion is metabolized via the dismutation reaction to yield $H_2O_2$ which could be further converted by another pathway which involves an iron-catalyzed reaction converting iron into hydroxyl radical (OH), known as Fenton-like reaction. Adapted from biology.plosjournals.org

### 1.2.2 Inflammation

There is a growing body of evidence suggesting that inflammation plays an important role in the initiation and progression of cardiovascular diseases (Schiffrin, 2002). Epidemiological studies have revealed strong associations between biochemical markers of systemic inflammation and the future risk for symptomatic cardiovascular disease (Granger et al., 2004). Several studies have provided convincing evidence that the known risk factors for cardiovascular diseases (hypertension, hypercholesterolemia, and smoking) can elicit both an inflammatory in the vascular system (Singh et al., 2005). The designation of cardiovascular disease as a chronic inflammatory process is supported by evidence that the risk factors for cardiovascular disease cause endothelial cells of the vasculature to assume an inflammatory phenotype (Kinlay et al., 2001). These activated endothelial cells characteristically exhibit oxidative stress and increased adhesiveness for circulating leukocytes (Hayashi et al., 1999), which are recruited to sites of inflammation by a highly coordinated and well-regulated process that involves the expression and/or activation of adhesion molecules on endothelial cells and circulating inflammatory cells (Fuller et al., 2003).

### 1.2.3 Ischaemia-Reperfusion Injury

Although the functional consequences of depriving tissue of its blood supply have been appreciated for many years, it has become apparent that reperfusion, the restoration of blood flow after a period of ischaemia, can place ischaemic organs at risk of further cellular necrosis, and thereby limit the recovery of function of the organ (Carden and Granger, 2000). It is widely recognized that the microvasculature, particularly the endothelial cells lining

microscopic blood vessels, is very vulnerable to the deleterious consequences of ischaemia and reperfusion (I/R) (Carden and Granger, 2000). Reperfusion of ischaemic tissues is often associated with microvascular dysfunction that is manifested as impaired endothelium-dependent dilation in arterioles, enhanced fluid filtration and leukocyte plugging in capillaries, and the trafficking of leukocytes and plasma protein extravasation in venules, which leads to the loss of microvascular integrity and decreased blood flow (Kupiec-Weglinski and Busuttil, 2005). Indeed, I/R-induced microvascular dysfunction has been described in most organs, and it is recognized as a potentially serious problem that is encountered during a variety of standard medical and surgical procedures, such as thrombolytic therapy, organ transplantation, coronary angioplasty, and cardiopulmonary bypass (Burns et al., 1998). Considerable research effort has been directed at elucidating the mechanisms underlying the patho-physiologic alterations associated with reperfusion of ischaemic tissues. Furthermore, a number of putative etiological factors have been identified. These include free radical generation (Carden and Granger, 2000), alternation of calcium concentration (intracellular calcium overload) (Dhalla et al., 1999) ATP depletion (Lavitrano et al., 2004) and apoptosis (Burns et al., 1998) (Fuller et al., 2003).

### 1.2.4 Ischaemic Heart Disease

Ischaemic heart disease (IHD) is the generic designation of a group of closely related syndromes resulting from myocardial ischaemia-an imbalance between the supply (perfusion) and demand of the heart for oxygenated blood (Ceconi et al., 2003). In more than 90% cases, the cause of myocardial ischaemia is reduction of coronary blood flow because of atherosclerotic coronary arterial

obstruction. In general, the clinical manifestation of IHD can be divided into four syndromes, myocardial infarction, angina pectoris, chronic ischaemic heart disease, and sudden cardiac death. The dominant influence in the causation of the IHD syndromes is diminished coronary perfusion relative to myocardial demand, owing largely to a complex dynamic interaction among fixed atherosclerotic narrowing of the coronary arteries, intraluminal thrombosis overlying a disrupted atherosclerotic plaque, platelet aggregation and vasospasm. Ischaemia results in several metabolic changes in cells, including decreased production of ATP (Lavitrano et al., 2004). Continued need for high-energy phosphate bonds results in further catabolism of ATP to ADP and ultimately to hypoxanthine, which accumulates in the ischaemic tissue, with reperfusion, the excess hypoxanthine is converted to xanthine by xanthine oxidase (Hamilton et al., 2004), this reaction generates large amounts of reactive oxygen species (ROS). The aftermath of this uninhibited release of ROS in the myocardium is activation of an inflammatory reaction, which is responsible for functional deterioration of myocardial performance during the immediate reperfusion period (Hamilton et al., 2004). The signaling pathway of these events is thought to involve activation of nuclear factor-KB (NF-KB) (Molavi and Mehta, 2004). Activation of NF-KB is then followed by modification of the expression of inflammatory mediator genes such as interleukin-6 (Singh et al., 2005), which determines the extent of myocardial inflammation, and thus outcome during the reperfusion period (Carden and Granger, 2000). Therefore, ROS seem to trigger a cascade of cellular events leading to inflammation and eventually cardiac injury (Kinlay et al., 2001).

### 1.2.5 Atherosclerosis

Oxidized lipids play an integral role in the pathogenesis of atherosclerosis, in which the production of ROS and nitrogen species in the vasculature leads to oxidation of low-density lipoprotein (LDL) (Kinlay et al., 2001). The responses of the endothelium, neutrophils and vascular smooth muscle cells to these oxidation products are thought to contribute to the development of the atherosclerotic process (Schiffrin, 2002). Endothelial dysfunction is thought to be one of the initial events in atherogenesis (Schiffrin, 2002), in addition, inflammatory cell infiltration and vascular smooth muscle cell migration along with oxidized LDL deposition generate fatty streaks and ultimately atherosclerotic plaques (Singh et al., 2005). Atherosclerosis, and particularly plaques responsible for acute coronary syndromes, characteristically contains inflammatory cells that play an important role in the pathogenesis of atherosclerosis (Schiffrin, 2002). Endothelial cells play an active role in the recruitment of inflammatory cells into the vessel wall, by producing cytokines and expressing cellular adhesion molecules (Kinlay et al., 2001). The NF-KB signal transduction pathway is a particularly important regulator of the transcription of a number of pro-inflammatory genes, including those that lead to the expression of adhesion molecules (Lakatta, 2003). NF-KB is a heterodimer containing two protein subunits that are normally held in an inactive state in the cytoplasm by the inhibitory component I-KB (Li et al., 2004). NF-KB is redox sensitive and activated by the degradation of its inhibitory component I-KB (Verma and Stevenson, 1997), a process that is accelerated by oxidized LDL cholesterol (Lakatta, 2003). Therefore, oxidative stress and inflammation play

an important role in the pathogenesis of atherosclerosis (Parthasarathy et al., 2001).

### 1.2.6 Hypertension

Elevated blood pressure (hypertension) affects both the function and the structure of blood vessels. Hypertension is a common health problem and it one of the most important risk factors in IHD (Chen et al., 2003). Endothelial dysfunction and remodeling of the vessel wall of large and small arteries is associated with hypertension (Schiffrin, 2002). These changes alter vascular function, aggravate high blood pressure, and may accelerate the progression of atherosclerosis (Carden and Granger, 2000). Activation of oxidative stress by angiotensin II is a key component of this process (Ceconi et al., 2003). Angiotensin II stimulates NADPH/NADH oxidase in endothelium, smooth muscle cells, and the adventitia of blood vessels to generate ROS, leading to endothelial dysfunction and inflammation (Granger et al., 2004). Up-regulation of endothelin-1, adhesion molecules, NF-KB, and other inflammatory mediators, as well as increased breakdown of NO, contribute to the progression of vascular disease and hypertension (Molavi and Mehta, 2004).

### 1.2.7 Overview of Heart Transplantation

Heart transplantation is a proven modality for the treatment of end stage cardiac disease (Demmy et al., 1997), however, an important determinant of transplantation outcome is the adequacy of organ preservation (Jahania et al., 1999). Currently, heart preservation is limited to 4 to 6 hours of cold ischemic storage, and the effectiveness depends to a great extent on the preservation solution (Muhlbacher et al., 1999), furthermore, longer periods of ischemia are

known to adversely affect survival (Demmy et al., 1997). This is in contrast to preservation of the liver and kidney, which have been successfully preserved for 24 to 36 hours, although graft function may be transiently compromised (St Peter et al., 2002). The principles of organ preservation are flushing, cooling and pharmacologic intervention (Muhlbacher et al., 1999). Currently, several preservation solutions are being used clinically; amongst them are the University of Wisconsin Solution (UW), Celsior and St. Thomas solution (Southard and Belzer, 1995). However, UW solution is certainly the most used preservation solution for livers, kidneys and pancreas with excellent clinical and experimental preservation data (McLaren and Friend, 2003). Currently, organs are kept in the preservation solutions (static cryopreservation) before being implanted into the recipient body; the cold storage minimizes cellular swelling and membrane pump mechanisms and therefore maintains cellular ATP (McLaren and Friend, 2003). Cold ischaemia and re-oxygenation during organ preservation leads to a variety of stresses, including expression of adhesion molecules, which can lead to inflammatory responses and compromised functions (Fuller et al., 2003). Furthermore, hypothermia slows biochemical reactions and metabolic rates; during hypothermia, most enzymes show a 1.5 to 2 fold decrease in activity for every 10 degrees Celsius decrease in temperature, which retards lysis of lysosomes and leads to the release of enzymes that eventually cause cell death (Jahania et al., 1999). The formulation of preservation solutions is based on hypothermic arrest of metabolism, supplementation of a physical and biochemical environment that maintains viability of the tissue during hypothermia, and minimizing the damaging effects of the IR injury (Demmy et al., 1997). Preservation solutions can be classified

into extracellular and intracellular, based on $Na^+$ and $K^+$ concentrations (Jahania et al., 1999). The main purpose of the ionic ingredients in the preservation fluid is to induce rapid myocardial cellular membrane depolarization by reducing the transmembrane $K^+$ gradient, which results in electrical and mechanical activity arrest of the cardiac tissue (Land et al., 1994; Michel et al., 2002) (Sasaki et al., 1999) (Ramella-Virieux et al., 1997).

## 1.3 Strategies to Mitigate Cardiovascular Disease

### 1.3.1 Preconditioning

It is generally thought that the damaging effects of I/R injury arise from the generation of ROS after reoxygenation, which initiate a cascade of deleterious cellular responses eventually leading to inflammation and apoptosis which eventually leads to organ failure (Garcia-Criado et al., 1997). Therapeutic strategies aimed at ameliorating I/R injury are focusing mainly on preventing the production of ROS and down-regulating the signal transduction cascades, and related pro-inflammatory genes (Sato et al., 2000). Among the most promising strategies is preconditioning (Baguneid et al., 2004). Preconditioning confers resistance to the subsequent deleterious stress that the graft is subject to during transplantation by inducing the expression of various endogenous protective mechanisms, including heat shock proteins (HSPs) (Okubo et al., 1999). To date, three types of preconditioning have been described, ischaemic preconditioning, thermal preconditioning and pharmacological preconditioning. Ischaemic preconditioning consists of a brief period of ischaemia followed by a short interval of reperfusion before the actual surgical procedure, which is characterized by a prolonged ischaemic stress (Peralta et al., 1997). In mouse

liver, ischaemic preconditioning confers dramatic protection against prolonged ischaemia via inhibition of apoptosis (Yadav et al., 1999). Thermal preconditioning, the elevation of the core temperature above normal before the injury, has been demonstrated to attenuate subsequent injury. Studies have suggested that the protective role of thermal preconditioning might be mediated by HSPs (McCormick et al., 2003). HSP induction after heat preconditioning improved the outcome in experimental models of renal grafts in rats (Redaelli et al., 2002b). It was shown that HSPs may inhibit renal tubular cell apoptosis by preventing the activation NF-KB and TNF-α production (Meldrum et al., 2003).

### 1.3.2 Modulation of Endogenous Cytoprotective Enzymes

Using pharmacological preconditioning to modulate the expression of intracellular pathways in organs or tissues can offer protection against oxidant-mediated injury; can be regarded as a novel strategy to combat different stress conditions that affect the cell (Lee and Surh, 2005). This strategy results in increase of cell defenses by inducing endogenous pathways, which are capable of restoring cellular homeostasis (Otterbein and Choi, 2000). HSPs, exemplify this concept because of their function as molecular chaperones and their high inducibility in stress conditions (Ryter and Choi, 2005). The induction of HSPs and other antioxidant genes in various stress conditions is being regarded as a refined stratagem by tissues to counteract a variety of patho-physiological states such as I/R injury (Redaelli et al., 2002a) and oxidative stress(Calabrese et al., 2003).

## 1.4 Endogenous Cytoprotective Machinery

Living cells are continually challenged by conditions which cause acute or chronic stress (Motterlini et al., 2002b). To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses which detect and control diverse forms of stress (Otterbein et al., 2003). Among these responses, are the heat shock proteins (HSP) (Calabrese et al., 2003) and phase II enzymes (Owuor and Kong, 2002).

### 1.4.1 Heat Shock Proteins (HSPs)

HSP have attracted a great deal of attention as a universal fundamental mechanism necessary for cell survival under a wide variety of toxic conditions (Redaelli et al., 2002b). In mammalian cells, HSPs synthesis is induced not only after hyperthermia, but also following alterations in the intracellular redox environment, exposure to heavy metals, amino acid analogs or cytotoxic drugs (McCormick et al., 2003). While prolonged exposure to conditions of extreme stress is harmful and can lead to cell death, induction of HSP synthesis can result in stress tolerance and cytoprotection against stress-induced molecular damage (Maines, 1997). The discovery of HSP has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear as possible candidates for novel cytoprotective strategies.

### 1.4.2 Phase II Enzymes

It was suggested that the metabolism of xenobiotics involves the actions of two families of enzymes, phase I and phase II (Williams, 1967). Phase I enzymes (such as cytochromes $P_{450}$) convert mild pro-oxidant toxins or electrophiles, to highly reactive electrophilic and hydrophobic toxins, that cause structural and

functional damage to the cell (Xu et al., 2005). However, Phase II enzymes protect cells against damage by electrophiles through multiple mechanisms, which include prompt conjugation of the phase I products with endogenous ligands such as glutathione (by glutathione *S*-transferases) and glucuronic acid (by UDP-glucuronosyltransferases), or via their anti-oxidant products which inactivates these agents resulting in the production of more water-soluble products that could be easily excreted (Talalay and Fahey, 2001). The phase II response is therefore emerging as a very important component of cellular defenses against oxidants (Talalay, 2005). Indeed, an ample literature now supports the view that induction of phase II enzymes is an important protective mechanism against the reactive oxygen species and essential for redox regulation (Moskaug et al., 2005). It is therefore of great importance to search for different pharmacological agents which possess intrinsic abilities to modulate the expression and the activity of these protective enzymes (Talalay and Fahey, 2001).

| Table 1-1: Phase II enzymes | | |
|---|---|---|
| **Inducible phase II proteins** | **Cytoprotective mechanisms** | **Reference** |
| Glutathione *S*-transferases (GSTA) | Conjugate with glutathione (GSH) and reduce alkyl, lipid, and DNA base hydroperoxides | [197]; [290] |
| UDP-glucuronosyl transferases | Conjugate with glucuronic acid | [146] |
| NADPH: quinone oxidoreductase (NQO) | Reduce quinones to hydroquinones | [54] |
| Haem oxygenase-1 (HO-1) | Generates antioxidants (bilirubin, CO) | [87] |
| Ferritin | Sequesters free ferrous iron | [107] |
| Superoxide dismutase | Reduces superoxide levels | [280] |
| Catalase | Reduces $H_2O_2$ levels | [240]; [119] |

Three cellular components are centrally involved in phase II gene regulation: Firstly, Antioxidant Response Elements (ARE), cis-acting promoters of transcription which are present in the upstream regions of many phase II genes (Chen et al., 2004). Secondly, Nrf2 (nuclear factor-erythroid-related factor 2), a basic leucine zipper transcription factor, binds in heterodimeric combination with other transcription factors to the ARE promoters, and signals enhanced transcription of phase II genes (Lee and Surh, 2005). And thirdly, Keap1, a repressor protein that is normally localized in the cytoplasm, where it is tethered to the cytoskeleton (Motohashi and Yamamoto, 2004), Keap1 binds to Nrf2 very tightly and is thereby largely retained in the cytoplasm, so that its activity is repressed (Alam et al., 1999). Inducers of phase II enzymes disrupt the Keap1-Nrf2 complex, thereby releasing Nrf2 for translocation to the nucleus and activation of the transcription of phase II genes (Kobayashi and Yamamoto, 2005).

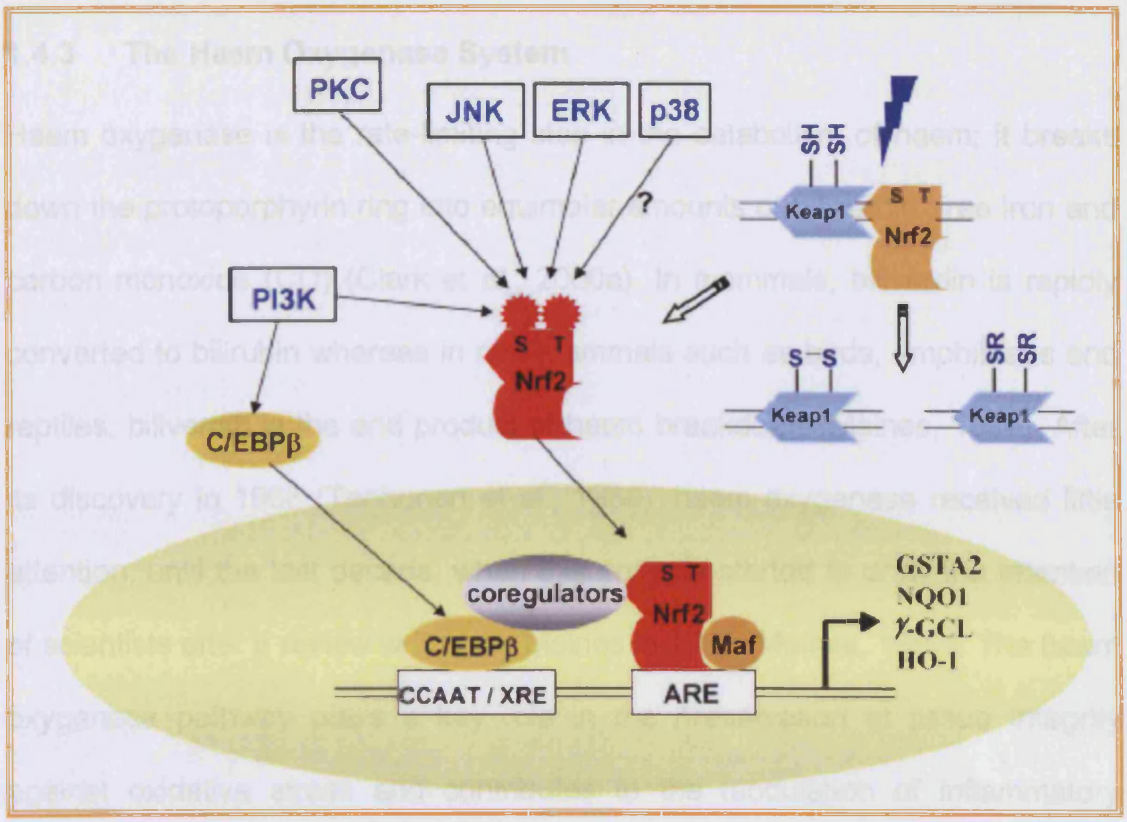



**Figure 1.4: Signalling pathways involved in the ARE-mediated gene transcription through activation of Nrf2.**

Keap1, as a cytoplasmic repressor, sequesters Nrf2 in the cytoplasm, thereby blocking the nuclear translocation of Nrf2 and subsequent transactivation of ARE. Alterations in cellular redox balance towards prooxidative status leads to the translocation of Nrf2 to the nucleus, where it binds to the ARE regulatory region of phase II genes. After translocation to the nucleus, Nrf2 may dimerize with a Maf proteins. The resultant heterodimer binds to the ARE and up-regulates the ARE-driven genes, including GSTA2, NQO1, g-GCL (gamma-glutamate cysteine ligase), and HO-1. Activated PKC may directly phosphorylate Nrf2. Three MAPKs, extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, may also stimulate the Nrf2 activation. Alternatively, PI3K can phosphorylate Nrf2, inducing its translocation to the nucleus. Some phase II enzyme inducers trigger the Nrf2 signal transduction by activating upstream kinases via phosphorylation and/or covalent modification/oxidation of cysteine thiol groups present in Keap1 (Lee and Surh, 2005).

### 1.4.3 The Haem Oxygenase System

Haem oxygenase is the rate-limiting step in the catabolism of haem; it breaks down the protoporphyrin ring into equimolar amounts of biliverdin, free iron and carbon monoxide (CO) (Clark et al., 2000a). In mammals, biliverdin is rapidly converted to bilirubin whereas in non-mammals such as birds, amphibians and reptiles, biliverdin is the end product of haem breakdown (Maines, 1997). After its discovery in 1968 (Tenhunen et al., 1969), haem oxygenase received little attention, until the last decade, when this enzyme started to draw the attention of scientists after a review written by Maines in 1993 (Maines, 1997). The haem oxygenase pathway plays a key role in the preservation of tissue integrity against oxidative stress and contributes to the modulation of inflammatory responses, acting in synchrony with other enzymatic systems that are involved in cytoprotection (Foresti et al., 2001) (Foresti et al., 2004). Recently, the crucial role of haem oxygenase in vascular biology was highlighted by the case of a child with haem oxygenase deficiency. In this patient, (who died at the age 6 years due to intracranial haemorrhage (Ohta et al., 2000)) haemolysis and endothelial cell injury were prominent features, furthermore, he suffered from severe growth retardation, persistent haemolytic anaemia characterized by marked erythrocyte fragmentation and intravascular haemolysis, and the presence of severe and persistent endothelial damage (Shibahara et al., 2002; Yachie et al., 1999). Similar features, including growth retardation, anaemia, iron deposition and vulnerability to stressful injury are all characteristics recently described in HO-1 null mice (Yachie et al., 1999). The clinical symptoms in this patient demonstrated the critical importance of HO-1 in iron metabolism and cytoprotection from oxidative damage (Yachie et al., 1999).

The catabolism of haem by haem oxygenase is the only biological process which is colorimetric, and can be noted as a gradual change in colour of a bruise. After receiving a blow to the skin, the colour of the skin becomes black or purple which is the colour of haem released from red blood cells after trauma, then black is gradually transformed to green, the colour of biliverdin, and finally to yellow, the colour of bilirubin (Figure 1.5) (Otterbein and Choi, 2000).

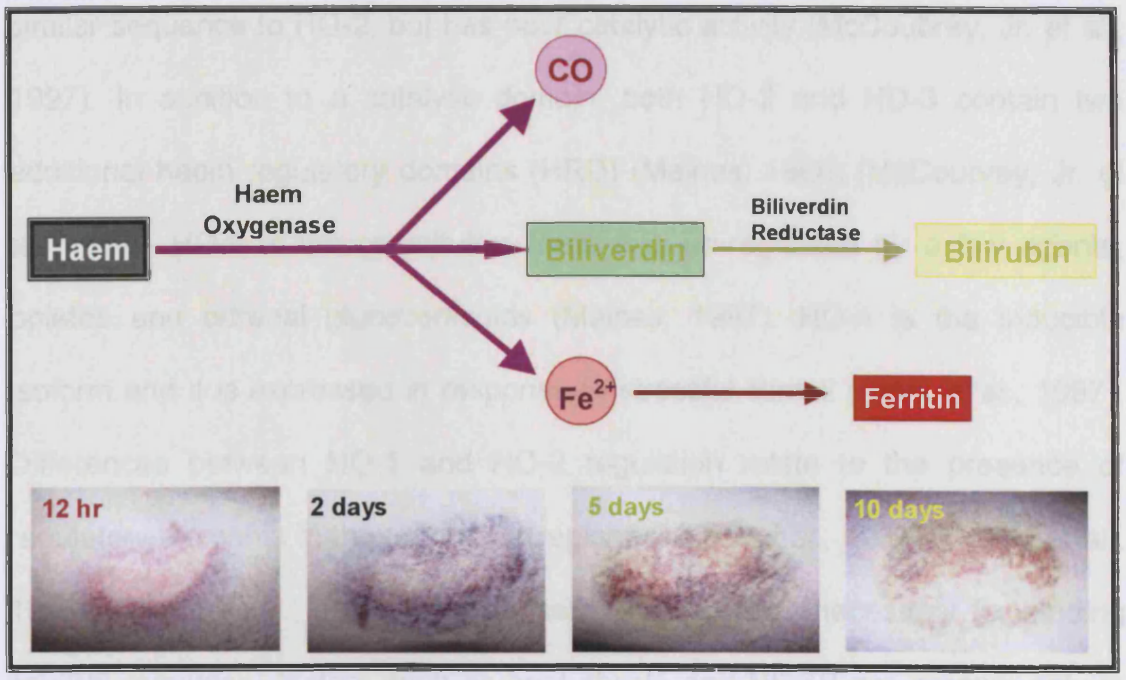



**Figure 1.5: The haem oxygenase reaction**

Haem oxygenase catalyzes the first and rate-limiting step in the degradation of haem, to yield equimolar quantities of biliverdin, CO, and free iron. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, and free iron is promptly sequestered into ferritin. Catabolism of haem is the only biological process in humans that is colorimetric; after receiving a blow to the skin, shortly thereafter a black or purple bruise would be observed. These are colours of haem, released into the dermis from pulverized erythrocytes. The black hue (haem) gradually transformed to green, the colour of biliverdin, and finally to yellow, the colour of bilirubin, the concluding product of this elegant enzymatic reaction (Otterbein and Choi, 2000).

To date, three isoforms of haem oxygenase have been identified to date haem oxygenase-1 (HO-1), haem oxygenase-2 (HO-2) and haem oxygenase-3 HO-3) (Maines, 1997), with molecular weights of approximately 32, 36 and 33 KD respectively (Table 1-2). The three enzymes are products of different genes and vary in their tissue distribution and regulation (Maines, 1997). Both HO-1 and HO-2 catalyze identical biochemical reactions, but differ in reaction rates, primary structure, and thermostability (Maines, 1988). In contrast, HO-3 has a similar sequence to HO-2, but has poor catalytic activity (McCoubrey, Jr. et al., 1997). In addition to a catalytic domain, both HO-2 and HO-3 contain two additional haem regulatory domains (HRD) (Maines, 1988) (McCoubrey, Jr. et al., 1997). HO-2 is the constitutive form; it is up-regulated by a few agents, opiates and adrenal glucocorticoids (Maines, 1997). HO-1 is the inducible isoform and it is expressed in response to stressful stimuli (Clark et al., 1997). Differences between HO-1 and HO-2 regulation relate to the presence of regulatory elements in their promoter regions (Muller et al., 1987) (Camhi et al., 1995) (Camhi et al., 1998). The consensus sequences necessary for binding several regulatory factors such as heat shock, and NF-KB are present only in the HO-1 promoter region whereas in contrast, a single functional glucocorticoid response element (GRE) is present in the promoter region of HO-2 (Liu et al., 2000).

| Table 1-2: Characteristics of the haem oxygenase isoforms | | | |
|---|---|---|---|
| **Properties** | **HO-1** | **HO-2** | **HO-3** |
| **Cellular localization** | Microsomes | Mitochondria | unknown |
| **Chromosomal localization** | 22 q 12 (Kutty et al., 1994) | 16p13.3 (Kutty et al., 1994) | unknown |
| **Molecular weight** | 32 KD | 36 KD | 33 KD |
| **Tissue distribution** | Liver, kidney, heart, vascular smooth muscle, endothelium, lung and brain | Nervous system<br>Blood vessels<br>Testes<br>Intestine | Thymus<br>spleen<br>heart<br>testes<br>brain |
| **Roles** | Cellular homeostasis, anti-inflammatory | Neural signalling and vascular regulation | unknown |
| **Regulation** | Haem<br>oxidative stress<br>heavy metals<br>nitric oxide<br>hypoxia | Glucocorticoids, opiates | unknown |
| **Reviewed from references (Kutty et al., 1994)** | | | |

The specific activity of haem oxygenase varies in different organs (Maines, 1997). The highest haem oxygenase activity is found in the spleen, testes and the brain (Maines, 1988). The spleen is the only organ in which, under normal conditions, HO-1 is the predominant form (Braggins et al., 1986). In the cardiovascular system, HO-2 is the predominant form expressed under normal conditions; HO-2 is normally expressed in the endothelial and smooth muscle layers of the blood vessels under stressful conditions, the expression of HO-1 increases dramatically, particularly in the atrioventricular node and in myocytes (Ewing et al., 1994). In the liver, the expression of haem oxygenase isozymes shows a developmentally-related expression as the total haem oxygenase activity (which is mainly due to the increased expression of HO-1 protein) in the liver decreases as the newborn matures; on the other hand, HO-2 expression gradually increases as the animal matures (Sun and Maines, 1990). In the reproductive system, expression of HO-1 and HO-2 expression shows a cell-type specific pattern of expression, e.g. HO-2 is expressed in germ cell lines, predominantly in the mature spermatocytes whereas HO-1 is expressed in Sertoli cells (Ewing and Maines, 1995), the highest levels of HO-2 are found in the testis (Liu et al., 2000).

The products of the HO-1 pathway have been shown in many experiments to exert important biological and cytoprotective functions (McLaren and Friend, 2003) (Sato et al., 2001; Song et al., 2003). In particular, HO-1 has important antioxidant and anti-inflammatory functions (Alcaraz et al., 2003). The beneficial effects of HO-1 induction occur via several postulated mechanisms. Firstly, increased HO-1 activity results in the degradation of the haem moiety, which is per se a pro-oxidant molecule and potentially toxic (Balla et al., 1991).

Furthermore, HO-1 results in the generation of bilirubin, which possesses antioxidant properties which are involved in scavenging peroxy radicals and exerting inhibitory effects on lipid peroxidtaion (Llesuy and Tomaro, 1994; Stocker et al., 1987b). In addition, HO-1 produces CO, which has anti-apoptotic and anti-inflammatory actions as well as vasoregulatory activity (Clark et al., 2003) (Figure 1.6). Furthermore, HO-1 system also plays an important role in the homeostasis of iron by stimulating the up-regulation of ferritin; the storage protein for iron, endothelium may be protected from oxidant damage through ferritin, the iron chelator, which is co-induced with HO-1 (Balla et al., 1992).

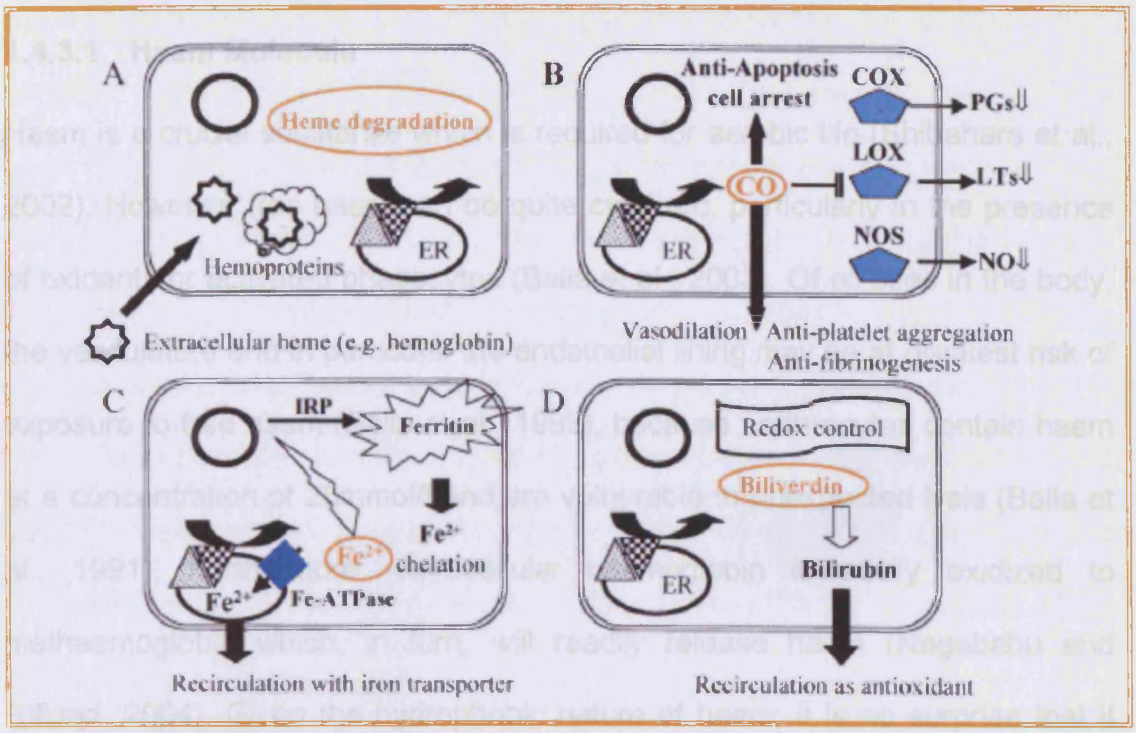



**Figure 1.6: The mechanisms of cytoprotection of the HO-1 system**

HO-1 (checkered diamond) degrades haem to produce three products: Fe2, CO, and biliverdin, in cooperation with cytochrome P-450 reductase (shaded diamond). (A) Haem is derived from both extracellular and intracellular compartments. (B) CO not stimulates leads to vasodilatation or prevention of platelet aggregation, induces cell arrest and inhibits apoptosis. CO also interferes with cyclooxygenase (COX), lipoxygenase (LOX), and NO synthase (NOS) activity, resulting in reduction of NO and prostaglandins (PG)–LT. (C) $Fe^{+}2$ released into endoplasmic reticulum (ER) by Fe-ATPase, may be redistributed or neutralized in ER. (D) Biliverdin, rapidly reduced to bilirubin by biliverdin reductase, is distributed in both intracellular and extracellular compartments. ROS can be scavenged by bilirubin to maintain redox status and protect cells from oxidative stress. Adapted from (Katori et al., 2002a)

### 1.4.3.1 Haem Molecule

Haem is a crucial substance which is required for aerobic life (Shibahara et al., 2002). However, free haem can be quite cytotoxic, particularly in the presence of oxidants or activated phagocytes (Balla et al., 2003). Of all sites in the body, the vasculature and in particular the endothelial lining may be at greatest risk of exposure to free haem (Balla et al., 1993), because erythrocytes contain haem at a concentration of 20mmol/l and are vulnerable to unexpected lysis (Balla et al., 1991). Furthermore, extracellular haemoglobin is easily oxidized to methaemoglobin which, in turn, will readily release haem (Nagababu and Rifkind, 2004) .Given the hydrophobic nature of haem; it is no surprise that it easily crosses cell membranes and can synergistically enhance cellular oxidant damage (Maulik et al., 1996).

### 1.4.3.2 The Catalytic Pathway of Haem Oxygenase

Haem oxygenase cleaves the substrate haem to form biliverdin, water, CO, and iron (Foresti and Motterlini, 1999). Haem degradation can also be carried out by other proteins, such as xanthine oxidase and NADPH-cytochrome P-450 reductase (Guengerich, 1978). In this case the end products of these enzymatic reactions are not biliverdin and CO, but a mixture of pyrrolic complexes (Shibahara et al., 2002). The haem oxygenase reaction requires NADPH: cytochrome P-450 reductase, which reduces the ferric haem complex, three molecules of oxygen and at least seven electrons provided by NADPH-cytochrome-P450 reductase (Maines, 1997). The haem oxygenase system is a mixed-function oxidase, for each mole of oxygen consumed in the reaction, one mole of $H_2O$ is produced (Kikuchi et al., 2005). In each mono-oxidation cycle,

the reduced iron binds molecular oxygen that accepts the electron from NADPH, the first cycle forms hydroxyl-haem, the second cycle forms verdohaem and thus breaks the α methane bridge carbon which will be released as CO, and the third cycle forms ferribiliverdin IX (BV-Fe III) α complex (Maines, 1997). An additional NADPH-dependent reduction of the (BV-Fe III) is needed before the dissociation of ferrous iron from biliverdin, then NADPH biliverdin reductase reduces the water soluble biliverdin to the hydrophobic bilirubin. The released ferrous iron is then chelated by apoferritin and stored in a ferric state within the ferritin molecule or transported to the bone marrow (Balla et al., 1992).

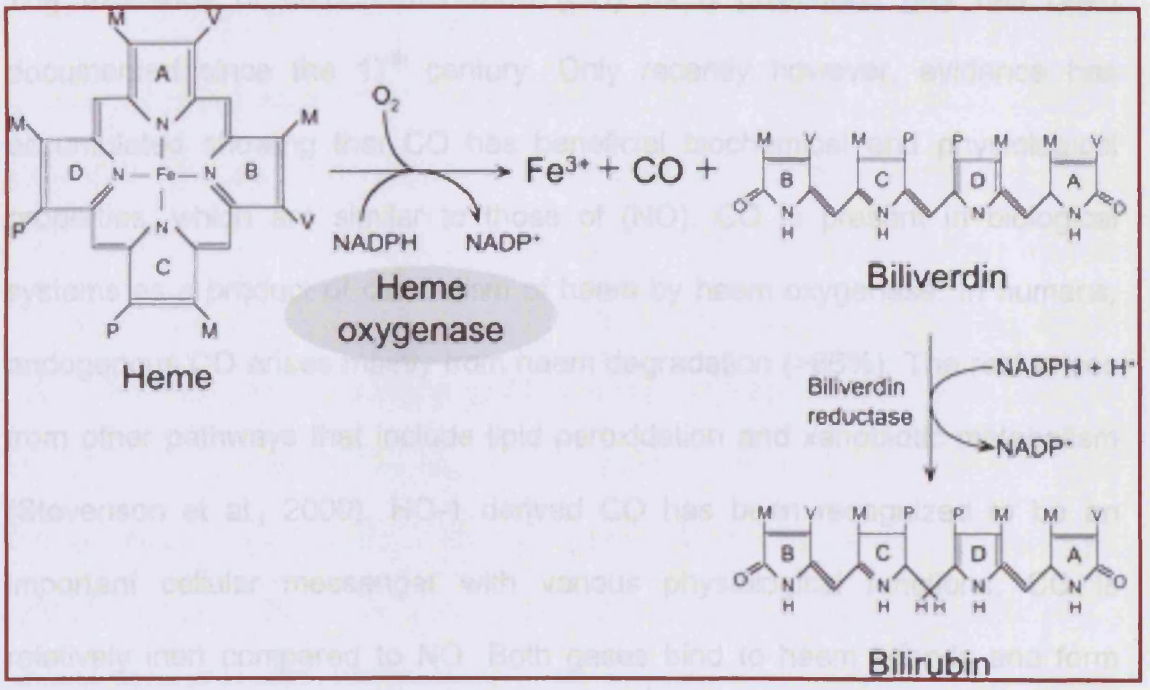



**Figure 1.7: Schematic diagram of haem catabolism via the haem oxygenase pathway.**

The haem oxygenase catalyzed reaction, haem (iron protoporphyrin IX) is cleaved between rings A and B by haem oxygenase to yield equimolar quantities of iron ($Fe^{2+}$), carbon monoxide (CO) and biliverdin. $O_2$ and NADPH are required for this reaction. Biliverdin is then converted to bilirubin by biliverdin reductase (Agarwal and Nick, 2000).

### 1.4.4 The Biological Roles of Haem Oxygenase Pathway

HO-1 up regulation is associated with inhibitory effects in various models of cardiovascular diseases; in inflammatory states (Laniado-Schwartzman et al., 1997), in graft rejection (Agarwal and Nick, 2000) and in atherosclerosis (Wang et al., 1998). HO-1 up regulation results in the inhibition of oxidative stress and apoptosis with significant reductions in inflammatory response including oedema, leukocyte adhesion and migration, and production of cytokines (Alcaraz et al., 2003).

#### 1.4.4.1 Carbon Monoxide

The existence of carbon monoxide (CO) as a poisonous gas has been documented since the $17^{th}$ century. Only recently however, evidence has accumulated showing that CO has beneficial biochemical and physiological properties, which are similar to those of (NO). CO is present in biological systems as a product of catabolism of haem by haem oxygenase. In humans, endogenous CO arises mainly from haem degradation (>86%). The rest arises from other pathways that include lipid peroxidation and xenobiotic metabolism (Stevenson et al., 2000). HO-1 derived CO has been recognized to be an important cellular messenger with various physiological functions. CO is relatively inert compared to NO. Both gases bind to haem ligands and form complexes with most haemoproteins at the haem iron centre, CO binds only to ferrous ($Fe^{2+}$) haem whereas NO binds both ferrous and ferric haem. NO, a free radical gas, participates in numerous redox reactions, including the reaction with superoxide anion radicals ($O_2^-$) to form peroxynitrite (Immenschuh and Ramadori, 2000). In contrast, CO is a stable non-radical molecule. Both CO and NO are involved in neural transmission and modulation of blood vessel function,

including smooth muscle relaxation, and inhibition of platelet aggregation. CO reacts exclusively with haem and/or iron and thus can accumulate in cells to levels much higher than those of NO (Soares et al., 2001). Adding to this many cells have a constitutively high capacity to generate CO and/or can be induced in very high levels (Maines, 1997), mammalian tissue has a very high capacity to produce CO, and in some organs, such as the brain, it exceeds the production of NO (Maines, 1997).

The beneficial effects of CO were demonstrated in a number of experimental models, for example, in a model of acute lung injury (Otterbein et al., 1999) CO was found to be protective in rat orthotopic lung transplantation (Song et al., 2003), in another model, it protects against septic shock, (Yachie et al., 1999) and provides protection in atherosclerosis (Shi et al., 2000a) (Shi et al., 2000b). Data in the literature provide evidence for the beneficial effects of CO in heart transplantation, HO-1-derived CO prevents I/R injury associated with cardiac transplantation possibly based on its anti-apoptotic action (Akamatsu et al., 2004). In intestines, perioperative CO inhalation at a low concentration resulted in protection against I/R injury to intestinal grafts with prolonged cold preservation (Nakao et al., 2003). The anti-inflammatory activity of CO may underlie the protective effects of CO in graft rejection (Ke et al., 2001) (Ke et al., 2002) (Sato et al., 2001). Exposure to CO gas has been shown to exert potent anti-apoptotic effects *in vivo* and *in vitro*. *In vitro*, CO prevented TNF-α-induced apoptosis in murine fibroblasts (Petrache et al., 2000). Similarly, CO prevented oedema formation and apoptosis during reperfusion after cardiopulmonary bypass in pigs (Lavitrano et al., 2004).

### 1.4.4.2 Biliverdin, Bilirubin and Iron

Biliverdin and bilirubin are reducing species and hence potential antioxidants formed by the action of haem oxygenase and biliverdin reductase (Stocker, 2004). Biliverdin is rapidly converted to bilirubin by biliverdin reductase (Katori et al., 2002a). Biliverdin administration has been shown to be protective against various cellular stresses, such as ischaemic heart injury (Vachharajani et al., 2000). Bilirubin is the most abundant endogenous antioxidant in mammalian tissues and accounts for most of the antioxidant activity in human serum (Gopinathan et al., 1994), it forms a complex with serum albumin (Kapitulnik, 2004), this complex prevents the peroxidation of albumin-bound fatty acids(Stocker et al., 1987a), bilirubin also reacts with peroxyl radical and lipid peroxides (Stocker et al., 1987b). Furthermore, bilirubin has been shown to inhibit the adhesion of neutrophils elicited by I/R (Hayashi et al., 1999). Bilirubin formed by the activation of HO-2 protects neurons against oxidative damage injury (Dore et al., 1999). Furthermore, bilirubin administration was proved to be protective in a model of ischaemic heart injury (Clark et al., 2000b). The haem oxygenase pathway also releases free iron, which has two free electrons capable of generating hydroxyl radicals through Fenton chemistry (Winterbourn, 1995) once released, iron is rapidly sequestrated into the iron storage protein ferritin (Harrison and Arosio, 1996). The release of iron by haem oxygenase also results in the up-regulation of ferritin, which acts as a scavenger of iron (Otterbein et al., 2003).

### 1.4.5 The Role of HO-1 in Inflammation

Over-expression of HO-1 results in a significant reductions in inflammatory events including edema, leukocyte adhesion and migration (Alcaraz et al., 2003) and production of inflammatory cytokine (Otterbein et al., 2003). Modulation of signal transduction pathways by HO-1 or its products mediate the anti-inflammatory effects of this protein (Ryter and Otterbein, 2004). HO-1 exerts its anti-inflammatory actions by down regulation of pro-inflammatory cytokines (Terry et al., 1998) (Rizzardini et al., 1993) (Otterbein et al., 2000) and up-regulation of ant-inflammatory mediators (Willis et al., 1996) (Lee and Chau, 2002) (Abraham and Kappas, 2005). HO-1 deficiency in humans is associated with susceptibility to oxidative stress and an increased pro-inflammatory state with severe endothelial damage (Yachie et al., 1999).

### 1.4.6 Haem Oxygenase System in Transplantation

Increased HO-1 expression has beneficial effects in a number of transplantation models (Katori et al., 2002b). It is thought that HO-1 exerts its protective effects through its anti-apoptotic (Akamatsu et al., 2004), anti-inflammatory (Song et al., 2003) and antioxidant functions (Tsuchihashi et al., 2003). It was demonstrated that the expression of HO-1 is up-regulated in the vascular endothelium of transplanted organs (Soares et al., 1998). The reason for this is that the endothelium in the vasculature of the graft is exposed to high levels of free haem following transplantation (Sato et al., 2001). Haem released from haemoglobin and myoglobin from the erythrocytes or myocytes through haemolysis or necrosis which occurs after transplantation (Balla et al., 1993). Once released from these proteins, free haem become incorporated into the lipid bilayer of the cytoplasmic membrane, and act as a potent pro-oxidant

(Ryter and Tyrrell, 2000), probably related to the fact that iron which is in the core of the haem molecule becomes available to participate to the generation of free radicals through the Fenton reaction (Ryter and Tyrrell, 2000). The generation of free radicals initiates a group of signal transduction pathways that induce both the expression of pro-inflammatory and pro-apoptotic genes (Gueler et al., 2004), which results in the up-regulation of cytokines and other mediators of inflammation (Carden and Granger, 2000). These events lead to the deleterious effects that cause I/R injury and ultimately, transplant rejection (Gueler et al., 2004). Under these conditions, HO-1 up-regulation plays a major role in combating these cellular and tissue stresses (Akamatsu et al., 2004). Once up-regulated, HO-1 produces CO which exerts its anti-apoptotic and anti-inflammatory actions, along with bilirubin, which act as antioxidants, in addition to the clearance of the pro-oxidant haem (Akamatsu et al., 2004) (Kato et al., 2003) . Furthermore, HO-1 action on haem promotes the release of free iron, which in turn up–regulates ferritin (Harrison and Arosio, 1996). The anti-apoptotic effect of HO-1 can be mimicked by the administration of iron chelator desferoxamine (Brouard et al., 2000). CO exposure protected transplanted lungs (Song et al., 2003). Furthermore, HO-1 induction attenuated oxidative injury in a rat kidney ischaemia-reperfusion model (Maines et al., 1999). It has been shown that HO-1 induction resulted in a longer cold storage of the harvested liver and reduced apoptosis in the hepatic graft using a model of liver transplantation in the rat (Redaelli et al., 2002a). Furthermore, expression of HO-1 in rodent liver, heart, and kidney allograft correlates with long-term graft survival (Coito et al., 2002) (DeBruyne et al., 2000) (Avihingsanon et al., 2002) (Tullius et al., 2001).

## 1.5 Regulation of HO-1 Expression

Great strides have been made in defining cellular events that govern HO-1 regulation. Arguably, HO-1 gene transcription is activated by far a greater number of stimuli than that of any gene in fact, only a handful of agents, among them being its own products, reduce HO-1 transcription (Maines and Gibbs, 2005) (Kitamuro et al., 2003). The overwhelmingly vast number of stimuli that activate HO-1 reflects the presence of multiple response elements within its promoter that bind activated factors and the multiplicity of interactions between components of the cell signaling cascades (Wright, 2000). The cellular processes underlying HO-1 induction are complex and tightly regulated, however, one feature common to most of the stimuli that up-regulate HO-1 is a significant shift in cellular redox (Motterlini et al., 2002b).

### 1.5.1 Transcriptional Regulation of HO-1 Gene

Induction of HO-1 expression is regulated primarily at the gene transcription level (Alam, 1994). It is regulated by two upstream enhancers, E1 and E2 (Alam and Cook, 2003) (Alam, 1994), both enhancer regions containing multiple stress responsive elements (stRE) (Alam and Cook, 2003). The signal transduction pathways that mediate HO-1 induction remains only partially understood (Maines and Gibbs, 2005).

#### 1.5.1.1 Regulatory Elements of the HO-1 Gene Promoter

The induction of HO-1 is mediated through *cis*-regulatory DNA sequences located in the promoter region, known as antioxidant responsive elements (AREs) (Alam et al., 1995), which are regulated by Nrf2 (Alam et al., 1999). Nrf2 is a member of Cap'n'Collar transcription factor/basic leucine zipper b, and

plays a major role in the transcriptional regulation of anti-oxidant and detoxifying genes (Lee and Surh, 2005). The transcription factor Nrf2 was recently identified as a general regulator of one defense mechanism against electrophiles. Nrf2 regulates the inducible expression of a group of detoxifying enzymes, such as HO-1, the important role of Nrf2 in the stress-dependent induction of HO-1 is confirmed by the finding that HO-1 is not inducible in Nrf2-null mice (Chan and Kan, 1999). Using peritoneal macrophages from Nrf2-deficient mice, it was demonstrated that Nrf2 controls the expression of HO-1 (Ishii et al., 2000), the response to electrophilic agents was profoundly impaired in Nrf2-deficient cells (Ishii et al., 2000). When un-stimulated, Nrf2 is located in the cytosol by binding to Keap1(Itoh et al., 1999), upon stimulation by oxidant stress, the Keap1-Nrf2 complex uncouples, leading to the translocation of Nrf2 from the cytosol to the nucleus and bind to a specific DNA sequence, thereby potentiating the ARE response (Itoh et al., 1999), (Owuor and Kong, 2002).

#### 1.5.1.2 Repression of HO-1 Gene Expression

Bach1 and Bach2 are transcriptional repressors (Blouin et al., 1998). It has demonstrated that HO-1 is constitutively expressed at high levels in Bach1-deficient mice (Sun et al., 2002). Bach2 is present only in B lymphocytes and the brain (Blouin et al., 1998) and is a known antagonist of the Nrf2- mediated induction of HO-1(Blouin et al., 1998) 78}. These findings were highlighted by the observation that expression of HO-1 mRNA and protein is significantly high in several organs of Bach–null mice, which was comparable to the basal levels of HO-1 detected in the spleen (an organ which normally exhibits high basal levels of HO-1 because of constant exposure the erythrocytes-derived haem) (Alam and Cook, 2003). On stimulation by haem, Bach1 is displaced, allowing

binding of Nrf2 to its DNA binding sites and consequently allowing the activation of different transcription factors (Sun et al., 2002). It is also worth mentioning that striking interspecies variations are noted in the regulation of HO-1 expression by hypoxia, heat shock, or interferon-gamma, each of which represses HO-1 expression in human cells, in human glioblastoma cells, interferon-γ repressed HO-1 mRNA expression (Takahashi et al., 1999). Hypoxia suppressed HO-1 mRNA expression in cultures of human umbilical vein cells, human astrocytes and human coronary arterial endothelial cells (Nakayama et al., 2000). Bach1 participates in hypoxia-inducible repression of human HO-1 expression (Kitamuro et al., 2003). Furthermore, it was shown that the regulation of HO-1 involves a direct sensing of haem levels by Bach1; expression of HO-1 m RNA is highly increased in human cells by haem (Yoshida et al., 1988).

#### 1.5.1.3 Role of MAPK Pathways

The mitogen-activated protein kinase (MAPK) signalling pathways play important roles in regulating gene expression in the cell (Kyriakis and Avruch, 2001). One of the major mechanisms for changes in gene expression appears to be through MAPKs, altering the activity of transcription factors and hence the transcription of their target genes (Torres, 2003). MAPK signalling pathways can either stimulate the translocation of transcription factors to the nucleus to promote their activity (Alam et al., 1999), or conversely, stimulate the export of transcription factors from the nucleus and hence facilitate their inactivation (Yang et al., 2003). MAPKs encompass a large number of serine/threonine kinases involved in regulating a wide range of cellular processes, including stress response and apoptosis (Sarkar and Li, 2004). There are four groups of

MAPK in mammalian cells; extracellular signal-regulated kinases (ERK), c-Jun N-terminal Kinases (JNK), P38 Kinases, and extracellular signal regulated kinase-5 ERK5; also called Big MAP kinase-1 (BMK1)(Kyriakis and Avruch, 2001). The ERK, JNK and p38 are activated through independent (sometimes overlapping) signalling cascades involving a MAPKK, MAPK kinase that is responsible for phosphorylation of the MAPK and a MAPK Kinase Kinase (MAPKKK) that phosphorylates and activates MAPKK (Torres, 2003) Figure1.8). MAPKs mediate their effects through phosphorylation of a wide range of transcriptions factors which are involved in many biological processes (Shimizu and Weinstein, 2005). ERK pathway is linked to the regulation of cell proliferation (Kolch, 2000), while, JNK and p38 are involved in the stress-related conditions (Sugden and Clerk, 1998). JNK and p38 pathways are involved in a wide range of stresses which include cytokines, heat shock and oxidative damage (Adler et al., 1999b). Furthermore, it has been demonstrated that p38 pathway is involved in curcumin-mediated induction of HO-1 (Balogun et al., 2003b). JNK activity is inhibited in un-stressed conditions, glutathione S-transferase (GST) binds to JNK and inhibits its activation, and this interaction is uncoupled under oxidative stress (Adler et al., 1999a) (Chen et al., 2001) (Ono and Han, 2000) (Ivanov and Ronai, 2000). NO induces HO-1 ERK and p38 (Chen and Maines, 2000), furthermore, HO-1 gene induction by quercetin in rat aortic smooth muscle cells involved p38 pathway (Lin et al., 2004) (Ryter et al., 2002) (Owuor and Kong, 2002). The anti-apoptotic effect of HO-1 in endothelial cells is mediated by the activation of p38 MAPK by CO (Brouard et al., 2000).

### 1.5.1.4 The Role of Phosphoinositide 3-Kinase Pathway

Lipid second messengers play a pivotal role in several cell signaling networks (Radeff-Huang et al., 2004), phosphoinositide 3-kinases (PI3Ks) generate specific inositol lipids that have been implicated in a plethora of cell functions (Neri et al., 2002). One of the best-characterized targets of PI3K lipid products is the serine/threonine protein kinase Akt. Recent findings have implicated Akt in cancer progression because it stimulates cell proliferation and suppresses apoptosis (Squires et al., 2003). Data in the literatures suggest that lipid molecules are important components of signaling pathways operating within the nucleus (Martin et al., 2004). PI3Ks, their lipid products, and Akt have also been identified as important cellular pathways and play a major role in the regulation of the phase II enzymes including HO-1 (Lee et al., 2001), PI3K leads to Nrf2 nuclear translocation and subsequent ARE activation (Nakaso et al., 2003) (Tiwari et al., 2004). Furthermore, PI3K has been implicated in the protective functions of the HO-1 pathway, for example, in a model of I/R injury (Fujimoto et al., 2004).

### 1.5.1.5 The Role of NO in the Regulation of HO-1

The signaling molecule NO, which is generated in mammals by a family of constitutive (nNOS and eNOS) and inducible (iNOS) NO synthase (NOS) enzymes (MacMicking et al., 1997; Maines, 1997), plays an essential regulatory role in a variety of physiological and pathophysiological processes that take place within the cardiovascular system (Chen et al., 2003). The distinctive biological activities evoked by NO can be explained by virtue of its nature as free radical, and by the reactivity of the NO group with the intracellular milieu (Motterlini et al., 2002b). HO-1 pathway is central to the regulation of many

physiological and pathophysiological processes, mainly due to its established function in haem catabolism (Otterbein et al., 2003). Furthermore, HO-1 is closely linked to NO molecule in the modulation of the tissue stress response, and is widely regarded as a key player in the restoration of vascular function under conditions of increased generation of ROS (Foresti et al., 2004). The HO-1 system may counteract the excessive production of NO and reactive nitrogen species (RNS), because of its high inducibility in response to NO and NO-related species, and therefore, actively participates in NO detoxification (Motterlini et al., 2002b). Induction of HO-1 by NO donors involves the activation of MAPK pathways (Chen and Maines, 2000) and is mediated through Nrf2 (Naughton et al., 2002).

#### 1.5.1.6 The Role of Intracellular Glutathione

Glutathione plays a critical role in cell physiology and is considered as a vital intra-cellular and extra-cellular protective anti-oxidant (Adler et al., 1999b). Glutathione exists in either a reduced (GSH) or oxidised (GSSG) form (Rahman and MacNee, 2000). It plays a vital role in cellular protection against oxidative stress and detoxification of exogenous and endogenous reactive heavy metals and electrophiles, cell cycle regulation and differentiation (Hammond et al., 2001).The GSH redox status is critical for various biological events (e.g. transcription of genes, modulation of redox-regulated signal transduction, regulation of apoptosis and inflammation (Rahman and MacNee, 2000). Changes in the cellular redox state may modify the activity of specific regulatory protein kinases and protein phosphatases which affect gene expression (Finkel, 1998). Glutathione redox homeostasis controls the level of ROS formed during cellular metabolism. It has been reported in a large number of studies that HO-1

gene expression is induced by stimuli that increase the production of ROS, including haem, $H_2O_2$ or by conditions that deplete cellular glutathione stores, including buthionine sulfoximine (BSO) which inhibits, the rate limiting step in the synthesis of glutatione. In rat brains, Maines *et al* demonstrated that glutathione depletion was associated with HO-1 induction in the brain (Ewing and Maines, 1993). Furthermore, it has been shown that scavengers of ROS, such as N-acetylcysteine inhibit oxidant-mediated HO-1 induction in many systems (Foresti et al., 1997) (Borger and Essig, 1998). Evidence suggests that both ultraviolet radiation and oxidizing agents potently induce HO-1 expression in human fibroblasts (Keyse and Tyrrell, 1987).

### 1.5.2 Regulation of Haem Oxygenase by Haem

Haem, a ubiquitous iron-containing compound, is present in large amounts in many cells and is also inherently dangerous, particularly when it escapes from intracellular sites (Balla et al., 1992). Free haem can be cytotoxic, especially in the presence of oxidants or activated phagocytes (Balla et al., 2003). The endothelium is at greatest risk of exposure to free haem, because erythrocytes contain high concentrations of haem (Maines, 1997), furthermore, extracellular haemoglobin is easily oxidized to methaemoglobin, which in turn releases free haem, which then (because of its hydrophobic nature) can easily cross the endothelial cell membrane and act to enhance cellular oxidant damage (Harrison and Arosio, 1996). Once within the cell, haem can promote oxidative damage directly by the release of iron, which, if in excess is toxic via the production of ROS by Fenton chemistry (Immenschuh and Ramadori, 2000).

## 1.6 Biological Effects of Dietary Phenolic Compounds: Relevance to Cardiovascular Disease

Plants have played a central role in the prevention and treatment of diseases since prehistoric times (Talalay and Talalay, 2001). Furthermore, a significant number of many of the drugs which are clinically used today are of plant origin (Table 1-3) (Talalay and Talalay, 2001). Recently, considerable attention has been focused on identifying dietary and medicinal phytochemicals that have intrinsic cytoprotective properties (Frusciante et al., 2000). Polyphenolic compounds form a major part of the dietary antioxidant capacity of fruits and vegetables (Blomhoff, 2005), and include Resveratrol (red wine) (Shimizu and Weinstein, 2005), curcumin (curry) (Motterlini et al., 2000b), chalcones (apples) (Tsao et al., 2003) and flavonoids (Lee et al., 2003). Flavonoids are the most abundant polyphenols in our diets and are found in fruits, vegetables, nuts, wine and tea (Kim et al., 2004). Polyphenolic compounds are receiving interest from scientists because epidemiological studies have suggested an association between the consumption of polyphenol-rich foods or beverages and the prevention of diseases such as cancer (Menon et al., 1999) (Ruby et al., 1995) (Talalay and Fahey, 2001), inflammatory diseases (Salh et al., 2003), neurodegenerative disorders (Lim et al., 2001), and cardiovascular diseases (Suzuki et al., 2001). The basic structure of flavonoids allows for a multitude of hydroxyl substitution patterns in the benzene rings, which give flavonoids the ability to scavenge ROS (Blomhoff, 2005).

| Table 1-3 : Important drugs that are of plant origin | | |
|---|---|---|
| **Drug** | **Plant origin** | **Clinical application** |
| **Digitalis** | *Digitalis purpurea* | congestive heart failure |
| **Colchicine** | *Colchicum autumnale* | Gout |
| **Opium** | *Papaver somniferum* | Analgesic |
| **Quinine** | *Cinchona* | Malaria |
| **Vinblatine** | *Madagascar periwinkle* | Cancer |
| **Etoposide** | *Mayapple rhizome* | Cancer |
| Reviewed from reference (Talalay and Talalay, 2001) | | |

### 1.6.1 Phytochemical-Regulated Signal Transduction Pathways

(Shimizu and Weinstein, 2005) Several mechanisms have been implicated in the cytoprotective properties of phytochemicals, including, scavenging of oxygen radicals and other highly reactive compounds (Blomhoff, 2005) and induction of detoxifying enzymes, such as phase II enzymes (Moskaug et al., 2005). Recent studies have emphasized a third mechanism, i.e. the ability of several phytochemicals to modify the activities of various receptor tyrosine kinases (RTKs) and related downstream pathways of signal transduction (Frigo et al., 2002), and thereby altering the expression of genes involved in inflammation, cell proliferation, and apoptosis (Shimizu and Weinstein, 2005) (Figure1.8). The activation of membrane-associated RTKs located at the cell surface by specific ligands (growth factors and cytokines) plays an important role in the control of many cellular processes (Kyriakis and Avruch, 2001). MAPK pathway is a downstream effectors can be activated in response to a

wide variety of extracellular stimuli, it has been shown that several phytochemicals activate MAPK (Frigo et al., 2002). The PI3K/Akt pathway is another important effector of the RTKs (Avruch et al., 2001), which appears to play a critical role in cell survival (Kim et al., 2005). The transcription factors AP-1 and NF-KB are critical downstream effectors of MAPK and PI3K/Akt signaling pathways, respectively (Schabbauer, 2004).

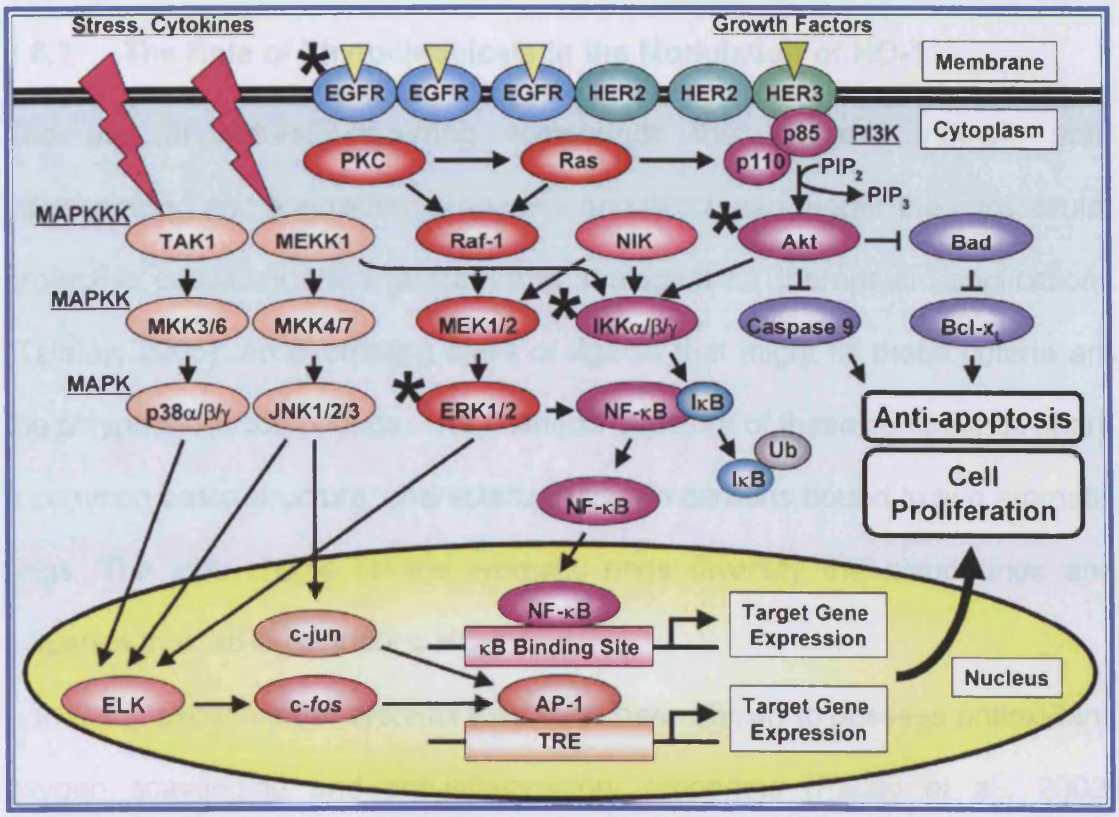


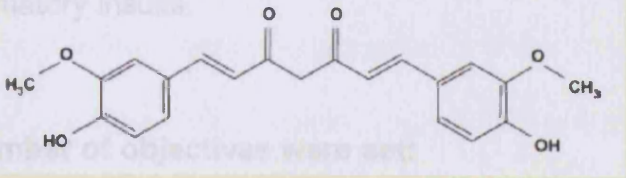
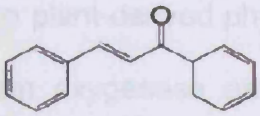
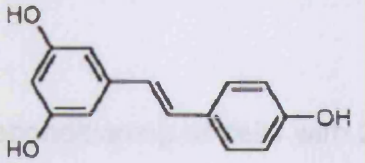
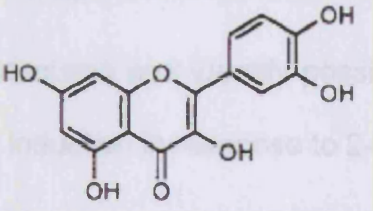

**Figure1.8: Signal transduction pathways involved in the function of phytochemicals**

A simplified scheme indicating how activation of of RTKs induces pathways of signal transduction that lead to activation of the transcription factors AP-1 and NF-KB. RTK are activated by specific ligands, thus leading to activation of their intrinsic tyrosine kinase and autophosphorylation of tyrosine residues. These activated RTKs then phosphorylate several downstream molecules, thus activating several signaling pathways. Activation of the small G protein Ras and effector proteins, such as Raf-1 and PI3K, stimulates several intracellular processes; which includes MAPKK, and MEK1/2 cascade which then phosphorylates the MAPK protein ERK1/2. The other MAPKs JNK and p38 are mainly activated by various stress events and cytokine stimuli. Once activated, MAPKs can activate a variety of transcription factors. PI3Ks are heterodimeric lipid kinases that are composed of a regulatory (p85) and a catalytic (p110) subunit. Among the RTKs, HER3 is the most efficient activator of PI3K because this receptor contains multiple binding sites for p85. Activation of PI3K causes the synthesis of the lipid PIP3, which activates downstream pathways that involve Akt, which enhances cell survival. Akt play roles in phosphorylation and activation of the kinase IKK. Activated IKK phosphorylates I-KB, which triggers the degradation of I-KB. The loss of I-KB enhances NF-KB activity through activation of MEK1/2 and ERK1/2. Adatpted from (Shimizu and Weinstein, 2005).

### 1.6.2 The Role of Phytochemicals in the Modulation of HO-1

The use of naturally-occurring compounds that possess intrinsic anti-inflammatory, and antioxidant properties and which can trigger the intracellular protective cascades offers a promising stratagem for therapeutic applications (Talalay, 2005). An interesting class of agents that might fill these criteria are the polyphenolic compounds. The chemical structure of these compounds share a common basic structure, characterized by two carbons bound to two aromatic rings. The side chains on the aromatic rings diversify the compounds and influence their ability to induce HO-1 (

). In many experimental systems they have been shown to possess antioxidant, oxygen scavenging and anti-inflammatory properties (Pataki et al., 2002) (Woodman and Chan, 2004) (Alcaraz et al., 2004). HO-1 is a highly sensitive inducible protein that is involved in the protection of the cell against a wide variety of stress (Foresti et al., 2001). Thus, HO-1 can be regarded as a potential therapeutic target in a variety of disease conditions that are mediated by inflammation and oxidative stress caused by production of free radicals, such as, IHD, septic shock, atherosclerosis and carcinogenesis (Clark et al., 2000a; Scapagnini, 2004) and contributes to the modulation of inflammatory responses (Alcaraz et al., 2003) (Owuor and Kong, 2002; Willis et al., 1996). Therefore, the search for potent inducers of HO-1 pathway can be regarded as a novel approach for the development of therapeutic applications for different pathological states.

| Table 1-4: Phytochemicals as Inducers of HO-1 | | | |
|---|---|---|---|
| **Compound** | **Diet source** | **Chemical structure** | **Ref.** |
| **Curcumin** | tumeric |  | [186] |
| **Chalcone** | hops |  | [86] |
| **Resveratrol** | grapes |  | [55] |
| **Quercetin** | apples |  | [153] |

### 1.6.3 Hypothesis and Aims

**The hypothesis of this thesis is:**

Increased haem oxygenase activity and induction of HO-1 protein expression by polyphenolic compounds offer protection to cardiovascular tissue against oxidative stress and inflammatory insults.

**To verify this theory a number of objectives were set:**

**(i)** Establish that two plant-derived phytochemicals, 2-HC and curcumin activate the haem oxygenase pathway in different cardiovascular tissues, i.e., vascular endothelial cells, macrophages and cardiac cells.

**(ii)** To verify that preconditioning of cells with 2-HC and curcumin protect cardiovascular cells against inflammation and oxidative stress.

**(iii)** Dissect the mechanisms and identify possible molecular targets that lead to the HO-1 induction in response to 2-HC.

**(iv)** Utilise CO, an end product of the haem oxygenase pathway, as an effective means to counteract inflammatory stress.

# 2 MATERIALS AND METHODS

## 2.1 Reagents and Solutions

Haemin (ferriprotoporphyrin IX chloride) and tin protoporphyrins (SnPPIX) were obtained from Porphyrin Products INC (Logan, Utah, USA). 2-HC was purchased from INDOFINE chemical company INC (Belle Mead, NJ 08502). Polyclonal antibodies for HO-1 were purchased from (Stressgen Biotechnologies Corporation, Victoria, BC, Canada), polyclonal antibodies for iNOS, NF-KB and Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other reagents were purchased from Sigma unless otherwise specified. Celsior solution was obtained from IMTIX SANGSTAT (Lyon, France). Stock solutions of different reagents were prepared in different solvents according to manufacturers' instructions. Curcumin and 2-HC were prepared in ethanol. Lipopolysaccharide (LPS–E. coli serotype 026:B6) was obtained from Sigma and the stock solution was prepared in phosphate buffer solution (PBS). CO-Releasing Molecules (CO-RMs) are synthesized on regular basis (in house) by collaborators of our group, (CORM-43 and CORM-319) were obtained from Brian E. Mann, Department of Chemistry, University of Sheffield, Sheffield. CORM-43 and CORM-319 were freshly prepared as 10 mM stock solution in distilled water ($dH_2O$). CORM-43 and CORM-319 were inactivated by adding cell culture medium to the compounds and leaving the solution for 24 h at 37°C in a 5% $CO_2$ humidified atmosphere to liberate CO. The inactive CORM-43 and CORM-319 (iCORM-43 and iCORM-319) solutions were finally bubbled with nitrogen to remove the residual CO present in the

solution. Haemin was dissolved in 0.1 M NaOH and 0.01M PBS pH 7.4. SnPPIX was prepared by dissolving the compound in 0.1M NaOH and $dH_2O$.

## 2.2 Cell Culture

### 2.2.1 Preparation and Maintenance of Cells

In this thesis, commercially available cell lines were used instead of a primary cell lines because of their convenience, and because the primary cultures are of mixed nature, and have a limited culture lifespan and have potential contamination problems. In contrast, the cell lines are more stable and less labour intensive to maintain. Admittedly, the advantages of primary cultures are that the cells have not been modified in any way (other than by enzymatic or physical dissociation by trypsin). The following cell lines were used in the experiments documented in this thesis: RAW 264.7murine macrophages and human cardiomyocytes (Girardi) were purchased from European Collection of Cell Cultures (ECACC) (Salisbury, Wiltshire, UK). Bovine Aortic Endothelial Cells (BAEC) were purchased from Coriell Cell Repositories (Camden, NJ, U.S.A.). The cells were received in the frozen state, in a 1 ml vial containing a cryoprotectant (DMSO). Upon receipt of cells, they were quickly thawed in a water bath at 37°C, the medium containing the cryoprotectant was removed and replaced with fresh complete medium. The contents were transferred carefully to a 25 $cm^2$ cell culture flask (Sarstedt Ltd., Leicester, UK) containing 6 ml of complete medium. The flask was incubated at 37°C in an incubator in a 95% $O_2$, 5% $CO_2$ environment. Subsequent medium changes took place every two days. The cells were grown in specific culture medium as outlined in Table 2-1.

| Table 2-1: The culture medium of the different cell types | | | | | |
|---|---|---|---|---|---|
| **Cell type** | **Media** | **FBS (%)** | **L-glutamine (mM)** | **Penicillin-Streptomycin (U/ml)** | **Additives** |
| **Girardi** | DMEM | 10 | 3.5 mM | 100 | 1% NEAA |
| **Raw 267.4** | DMEM | 10 | 2 mM | 100 | Nil |
| **BAEC** | ISCOVES' | 10 | 2 mM | 100 | Nil |
| *NEAA = non essential amino acids | | | | | |

Foetal bovine serum (FBS, 10%) was added to the medium, Dulbecco's modified Eagle's medium (DMEM) or ISCOVES', in addition to 100 U/ml penicillin, 0.1mg/ml streptomycin and (2-3.5) mM L-glutamine (Fluka Biochemika). Confluent cells were incubated with different reagents in 75 $cm^2$ flasks or 24 well plates in a final volume of 10 ml or 1 ml, respectively, of fresh complete DMEM. Cells were grown in 75 $cm^2$ tissue culture flasks and kept at 37°C in a 5% $CO_2$ humidified atmosphere. For hypothermia experiments, cells were kept in a temperature-controlled incubator (Sanyo MIR-153) and flushed with gas composed of 21% $O_2$, 5% $CO_2$.

### 2.2.2 Subculturing of Cells

In order to maintain the cell line, cells were subcultured every 3-4 days till they reached about 70%-80% confluence. The old medium was discarded and replaced with 10 ml of warm sterile phosphate buffered saline (PBS) (10X) (Invitrogen Life Technologies Ltd.). After a brief wash the latter was removed and replaced with 4 ml of warm 0.25% (w/v) trypsin-0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) solution. The flasks were then placed in the incubator for few minutes to allow the trypsin to detach the cells from the base of the flask and each other. The contents of the flasks were transferred

carefully to individual 15 ml tubes containing 7 ml of warm (37°C) fresh DMEM. After centrifuging (500 xg) for 5 min at room temperature in a bench-top centrifuge (MSE Harrier 15/80, Sanyo Gallenkamp Plc., Leicestershire, UK). The supernatant was discarded and the pellets were resuspended gently in sufficient DMEM to prepare a new set of flasks, with a final volume 10 ml, in a 1 to 3 ratio. The flasks were returned to the incubator to allow the cells to grow. Cells were subcultured until certain passage number (25 for BAEC and RAW 264.7 macrophages, and 230 for Girardi cells), after which time a new batch of cells was subcultured.

### 2.2.3 Collection of Cells

For this procedure, all reagents and collecting tubes (15 ml) were kept on ice. At the end of an experiment, the medium was discarded and cells were washed with 10 ml of ice-cold non-sterile PBS (10X). After a brief wash the latter was discarded and replaced with 5 ml of ice-cold PBS (1X) (PBS 10 X diluted 1:10 with $dH_2O$). Cells were then gently scraped from the base of the flasks with a plastic scraper (Sarstedt Ltd.) The mixture was then transferred to pre-labelled collecting tubes. A further 5 ml of PBS (1X) was added to each flask to collect any residual cells and then transferred to its respective tube. The tubes were centrifuged (500 g) for 5 min at 4°C to pellet the cells, the supernatants were discarded. Cell pellets requiring haem oxygenase activity analysis were resuspended in 550 µl of phosphate buffer (2 mM $MgCl_2$ and 100 mM $KH_2PO_4$, pH 7.4). Samples for HO-1 protein analysis were resuspended in 150 µl of PBS (pH 7.4), containing 1% (v/v) Triton X-100 to lyse the cells. Samples for RNA extraction were suspended in PBS 1 X, samples were dissolved fully using a bench-top vortexor and stored at -80°C until required for analysis.

## 2.3 Cytotoxicity and Cell Metabolism Assays

### 2.3.1 Cell Metabolism Assay

The oxidized blue, non-fluorescent Alamar Blue is reduced to a pink fluorescent dye in the medium by cell activity. Likely to be by oxygen consumption through metabolism or as now being suggested, reduced by mitochondrial enzymes (Nakayama et al., 1997) but it is not known whether this occurs intracellularly, at the plasma membrane surface or just in the medium as a chemical reaction (Ahmed et al., 1994). Cell metabolism was determined using a colorimetric viability assay kit (Serotec, Kidlington, Oxford, U.K.). The Alamar Blue stock solution was diluted 1:10 (v/v) in the culture medium according to the manufacturers' instructions. Cells were subcultured in a 24-wells plate, at the end of the experiment, after cells had been treated with various reagents, the medium was washed, and 1000 ml of the Alamar Blue dilutions was added into each well and left in the incubator at 37°C for 5 hours, or until a colour change was seen. The absorption at 570 and 600 nm was read in a microtitre plate reader (VersaMax. Molecular Devices U.K), the relative metabolic activity was determined by subtracting the absorbance measured at 600 nm from the absorbance at 570 nm.

### 2.3.2 LDH Assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells; the normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid (Abou El Hassan et al., 2003). *In vitro* release of LDH from cells correlates with the amount of cell

death and membrane damage and provides an accurate measure of cell membrane integrity and cell viability (Baudin et al., 1996). Extracellular LDH activity was measured using a cytotoxicity detection kit (Roche), according to manufacturer's instructions. At the end of the period of incubation, cell supernatant was collected; any cell residue is removed by centrifugation at 250 g. The reaction mixture (which is composed of the catalyst and the dye solution) was then added to the cell-free supernatant, incubated for 15 minutes, and then the absorbance was measured at 490 and 600 nm. Cellular LDH activity was determined after lysis of the cells with Triton X-100 (1% in DMEM at 25°C). Released LDH was expressed as percentage of total LDH activity.

### 2.3.3 Trypan Blue Assay

Trypan Blue is a dye, its reactivity is based on the fact that the dye is negatively charged and does not interact with the cell unless the membrane is damaged (Carpentier et al., 1981). Therefore, all the cells which exclude the dye are viable (Filman et al., 1975), cells were seeded in 6-well plates at $1 \times 10^4$ cells/well, and then after each treatment, cells were trypsinized, and centrifuged to produce cell pellet. Cells were suspended in culture medium and pipetted up and down several times to ensure a uniform cell suspension.1:1 dilution of the cell suspension and Trypan Blue solution (0.4% in PBS) was prepared (cell suspension combined with the Trypan Blue solution). To obtain an accurate cell count, a uniform suspension containing single cells is necessary. The cell suspension was pipetted up and down in the tube 4-5 times. After 1–2 min incubation with Trypan Blue, the number of dead cells, which retained the dye, was compared to the total number to calculate the percentage of dead cells, using a haemocytometer. Both the counting chambers of the haemocytometer

were loaded with the 10 µl of the diluted cell suspension using a micropipette and tips, cell suspension was allowed to fill the space by capillary action. The number of viable cells harvested from each 6-well plate was obtained by the following equation: UC × D × $10^4$/SQ, where UC = unstained cell count (viable cells), D = dilution of the cell suspension, and SQ = number of squares of the haemocytometer counted. The viability percentage of the cell population of each 6-well plate was obtained by the following equation: UC/TC × 100, where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells). {Freshney, R. (1987) Culture of Animal Cells: A Manual of Basic Technique, p. 117, Alan R. Liss, Inc., New York}.

## 2.4 Biochemical Assays

### 2.4.1 Protein Determination in Cells

The DC Protein Assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK) was used to determine protein concentration. A standard curve was prepared using serial dilutions (0 to 2.8 mg/ml) of a bovine serum albumin (BSA) standard (Bio-Rad Laboratories Ltd.), prepared in triplicate, in phosphate buffer (pH 7.4). The absorbance readings at an optical density of 750 nm ($OD_{750}$) were plotted against their respective protein standard concentrations (Figure 2.1) for a representative plot. The procedure for protein determination in cell and tissue samples was performed as follows. A 100 µl aliquot of the cell suspension and a blank of phosphate buffer or PBS Triton X-100 were added to 10 ml polystyrene tubes (Sarstedt Ltd.). When tissue samples were used, the aliquot was diluted 1:10 in phosphate buffer (pH 7.4). To each tube, 500 µl of A' (20 µl of Reagent S per ml of Reagent A) was added, then vortexed briefly without frothing. Next, 4 ml of Reagent B was added to

each tube, mixed well then left at room temperature for 20 min before reading the $OD_{750}$ against the blank using an UVikon 810P spectrophotometer (Tegimenta AG, Switzerland). A numerical value (mg/ml) for the protein concentration in the unknowns was derived by multiplying the respective $OD_{750}$ by the slope of the standard curve (Figure 2.1). Serial dilutions of bovine serum albumin (BSA) (0-2.8 mg/ml) were prepared. The OD750 was plotted against its respective protein concentration.

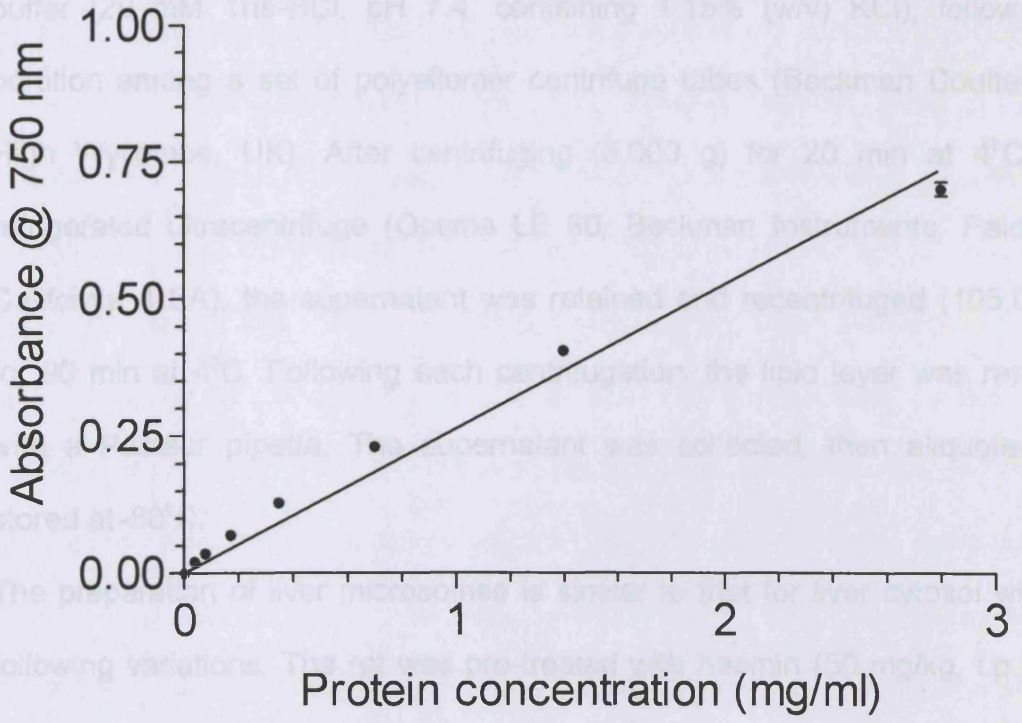



**Figure 2.1: Representative standard curve for protein determination**

$$OD_{750} \times \text{Slope of standard curve}$$

**Equation 2-1: Calculation of protein concentration**

### 2.4.2 Preparation of Liver Cytosol and Liver Microsomes

A male Sprague Dawley rat (250-300 g) was used as a source of liver cytosol, following sacrifice by anaesthetization with phenobarbitone Lethobarb® sodium BP (500 µl, i.p.) and cervical dislocation, liver cytosol was prepared as previously described (Motterlini et al., 1996). A 50 ml syringe was used to perfuse cold (4°C) buffer (1.15% (w/v) KCl) through the liver lobes to remove any residual blood, while clotted tissue was discarded. The organ was weighed, finely chopped with scissors and homogenised in 2-3 volumes of homogenising buffer (20 mM Tris-HCl, pH 7.4, containing 1.15% (w/v) KCl), followed by partition among a set of polyallomer centrifuge tubes (Beckman Coulter Ltd., High Wycombe, UK). After centrifuging (5,000 g) for 20 min at 4°C in a refrigerated ultracentrifuge (Optima LE 80, Beckman Instruments, Palo Alto, California, USA), the supernatant was retained and recentrifuged (105,000 g) for 90 min at 4°C. Following each centrifugation, the lipid layer was removed with a Pasteur pipette. The supernatant was collected, then aliquoted and stored at -80°C.

The preparation of liver microsomes is similar to that for liver cytosol with the following variations. The rat was pre-treated with haemin (50 mg/kg, i.p.) 24 h beforehand to induce haem oxygenase activity. The lobes were perfused with cold (4°C) saline (0.9% (w/v) NaCl) and homogenised in 5 volumes of sucrose solution (0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4) then centrifuged. After the last centrifugation step, the resulting microsomal pellet was resuspended gently in 1 ml of phosphate buffer (pH 7.4) and followed by storage at -80°C to be later used in the haem oxygenase activity.

### 2.4.3 Determination of Haem Oxygenase Activity

Stock solutions of the assay reagents, i.e., glucose-6-phoshate (G6P) (20 mM), glucose-6-phoshate dehydrogenase (G6PDH) (50 U/ml) and NADPH (40 mM), were prepared with phosphate buffer (pH 7.4) and stored at -80°C until required. Haemin (2 mM) was prepared freshly for each assay as described in section 2.1. Rat liver cytosol and liver microsomes were prepared according to section 2.4.2. Samples were disrupted by three rounds of freeze-thawing (-80° to 37°C). To a set of glass tubes, which were kept on ice, the cell suspension (400 µl) was added to the reaction mixture (final volume, 900 µl) containing: phosphate buffer (pH 7.4), 25 µM haemin, 2 mM G6P, 0.5 U/ml G6PDH, 3 mg of rat liver cytosol, as a source of biliverdin reductase, and 0.8 mM NADPH. The negative and positive controls (rat liver microsomes) contained proportionate volumes of phosphate buffer (pH 7.4) instead of cell suspension. The tubes were vortexed and incubated in the dark for 1 h at 37°C. The reaction was terminated by addition of chloroform (1 ml), followed by thorough vortexing and centrifuging (200 g) for 5 min at room temperature until three distinct layers were formed. Using a quartz cuvette and the UVikon 810P spectrophotometer, the absorbance (at 464 nm and 530 nm) of the lower layer, which contains bilirubin (extinction coefficient (ε) for bilirubin in chloroform, 40 $mM^{-1}cm^{-1}$), was read against a blank of chloroform. Haem oxygenase activity was expressed as Pico moles of bilirubin formed/mg protein/h using Equation 2-2.

$$\frac{\text{pmoles bilirubin}}{\text{mg protein in 60 min}} = \left( \frac{\Delta OD\ (OD_{464} - OD_{530})}{40} \Big/ \text{mg protein} \right) \times 10^6$$

**Equation 2-2 Calculation of haem oxygenase activity**

### 2.4.4 Determination of Nitrite Levels

Determination of nitrite ($NO_2^-$) can give an indication of the level of NO produced by the cells. Cells were subcultured in 24-well plates, when confluent; cells were exposed to LPS 1 µg/ml in the presence or absence of different reagents. Nitrite production was determined as described previously (Sawle et al., 2005). At the end of the experiment, 50 µl of each serial dilution and 50 µl of medium from each well were transferred into a 96-well plate (NUNC A/S Kam strupvej 90, Roskilde, Denmark). 50 µl of Greiss reagent (125 mg of sulphanilamide and 12.5 mg of N-1-naphtyl ethylenemine dihydrochloride dissolved in 8.65 ml of $dH_2O$ and 1.35 ml HCl) was then added to the samples and to the serial dilutions of the standard. The wells were then shaken for 10 minutes on a shaker (AM69 microshaker, from Cooke microtiter system, ZUG, Switzerland ).The absorbance was read at 550 nm using a plate-reader (VERSA $_{max}$ microplate reader from Molecular Devices). To prepare the serial dilutions for a standard curve, 6.5 mg of sodium nitrite ($NaNO_2$) was dissolved in 10 ml of $dH_2O$ to give a 10 mM stock solution of $NaNO_2$. It was dissolved 1 in 10 in warm DMEM to give a 1 mM concentration of $NaNO_2$ and then serial dilutions of the 1 mM $NaNO_2$ were prepared with DMEM to give a final volume of 1 ml.

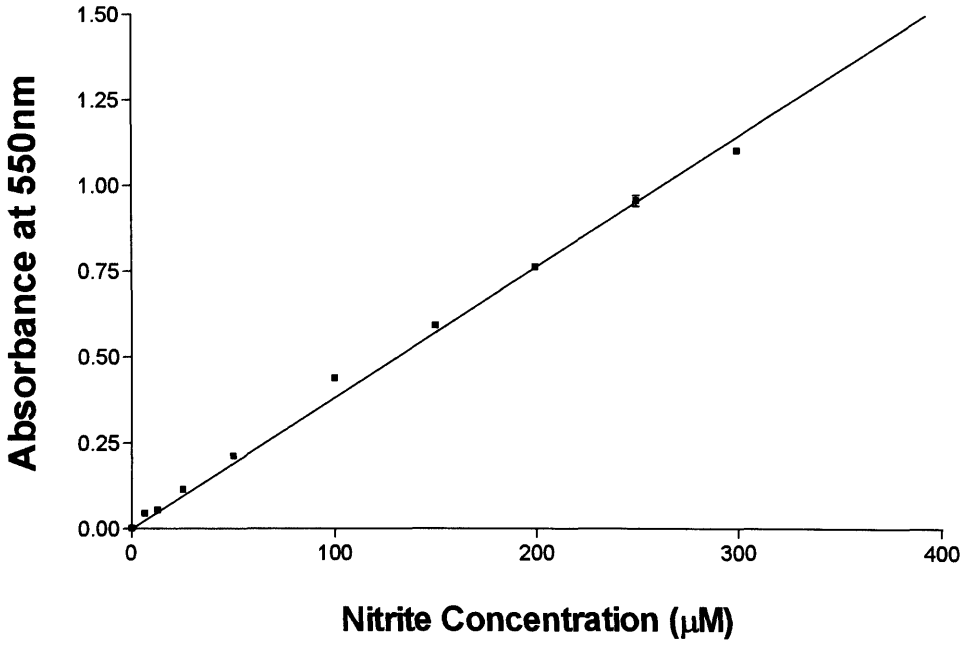



**Figure 2.2 : Standard curve for nitrite determination**

### 2.4.5 Determination of CO Release †

The release of CO from CORM-43, CORM-319 and their inactive forms was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) as previously described (Motterlini et al., 2002a). The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient=15.4 mmol/L-1 · cm-1). Myoglobin solutions (66 μmol/L final concentration) were prepared fresh by dissolving the protein in 0.04 mol/L phosphate buffer (pH 6.8). Sodium dithionite (0.1%) was added to convert myoglobin to deoxy-Mb prior to each reading. In contrast, CO released from both CORMs (CORM-43 and CORM-319) was quantified by adding aliquots of stock solutions (10 μL) of the carbonyl complex in water directly to the myoglobin solution. All the spectra were measured using a Helios α-spectrophotometer.

† Measurement of CO release was kindly performed by Mr. Philip Sawle.

## 2.5 Molecular Biology Procedures

### 2.5.1 Determination of TNF-α

The levels of TNF- α present in samples was determined using a commercially available kit from R&D Systems (Abingdon, U.K.). The assay was performed according to the manufacturers' instructions, as previously described (Sawle et al., 2005). Briefly, cell culture supernatants were collected immediately after the treatment and spun at 2000 g for 2 min to remove any particulates. 50 µl of the standard, the standard curve is demonstrated in Figure 2.3, and the samples was added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for mouse TNF- α and left to react for 2 hours, followed by a thorough wash using the wash buffer to remove any unbound antibody-enzyme reagent. Followed by incubation with 100 µl for 2 hours of mouse conjugate, 100 µl of the substrate solution was then added to each well and left for 30 minutes to allow the colour to develop. Finally, stop solution was added and the optical density was measured. The colour density was detected at 450 nm (correction wavelength 570 nm) as proportional to the amount of TNF-α produced. The standard curve was created using Prism software capable of generating a four parameter logistic (4-LP).

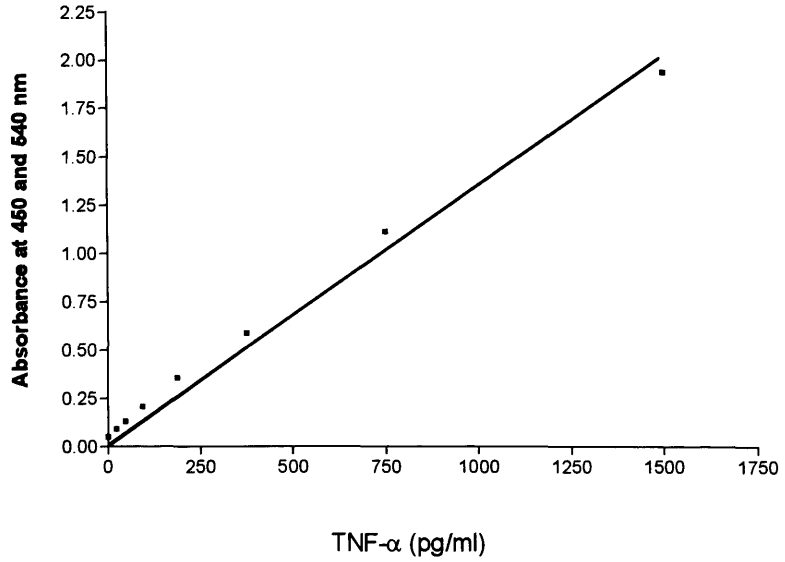



**Figure 2.3: Standard curve for the determination of TNF-α level**

### 2.5.2 Determination of Interleukin-10 (IL-10) Levels

IL-10 present in each sample was determined using commercially available kit from R&D Systems. The assay was performed according to the manufacturers' instructions. Briefly, cell culture supernatants were collected immediately after the treatment and spun at 2000 g for 2 min to remove any particulates. 50 µl of the standard, and the samples were added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for the mouse IL-10 and left to react for 2 hours, followed by a thorough wash using the wash buffer to remove any unbound antibody-enzyme reagent, followed by incubation for 2 hours with 100 µl of mouse conjugate, 100 µl of the substrate solution was then added to each well and left for 30 minutes to allow the colour to develop. Finally, stop solution was added and the optical density was measured. The colour density was detected at 450 nm (correction wavelength 570 nm) as proportional to the amount of IL-10 produced. The standard curve was created using Prism software capable of generating a four parameter logistic (4-LP).

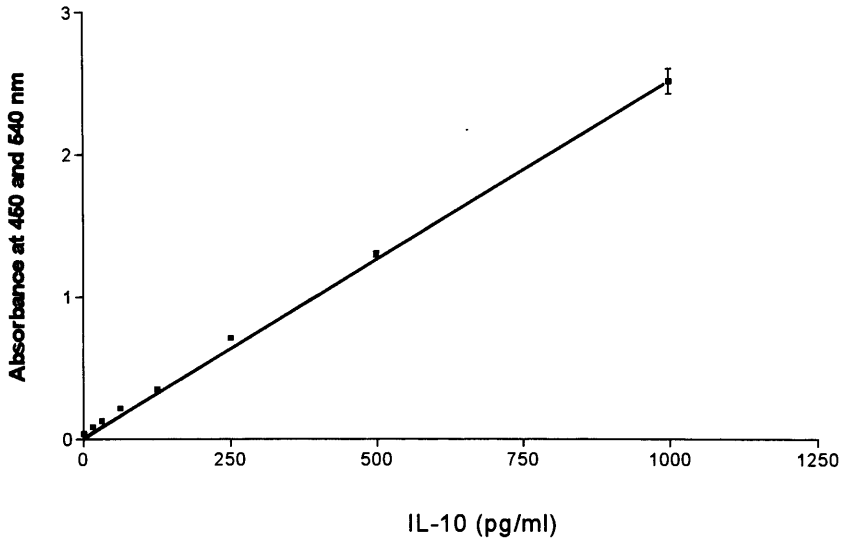



**Figure 2.4: Standard curve for the determination of IL-10 levels**

### 2.5.3 Preparation of Nuclear Extracts

For the determination of the translocation of Nrf2 and NF-KB, nuclear extracts were prepared as previously described (Balogun et al., 2003b) (Naughton et al., 2002). Cells were washed twice with PBS (1X), then harvested in 1 ml of PBS (1X) followed by centrifugation (800 g) for 3 min at 4°C. The resulting pellet was then resuspended carefully in 200 µl of cold Buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, to a final volume 950 µl, followed by 10 µl of 0.1 M dithiothreitol (DTT) and 40 µl of protease inhibitor cocktail. Next, pellets were placed on ice for 15 min to allow cells to swell, and then 15 µl of 10% Nonidet P-40 was added, followed by a 10 sec vortex-mix and centrifuging (800 g) of the resulting homogenate for 3 min at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 30 µl of cold Buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 µl of 0.1 M DTT and 40 µl of complete protease inhibitor cocktail. The pellet was placed on ice for 15 min and vortex-mixed for 10-15 sec every 2 min. After a final

centrifugation (15000 g) for 5 min at 4°C the supernatant, which contains the nuclear proteins, was retained and stored at -80°C freezer until further analysis of Nrf2 and NF-KB, β-actin was used as an internal control for loading, ß-Actin, once thought to be an exclusively cytoplasmic protein, is now known to have important functions within the nucleus (Olave et al., 2002). Nuclear ß-actin associates with and functions in chromatin remodeling complexes, ribonucleic acid polymerase complexes, and at least some ribonucleoproteins (McDonald et al., 2006), and therefore ß-Actin is suitable as a loading control for nuclear extracts (Ishii et al., 2000).

### 2.5.4 HO-1 and PI3K siRNA Transfection

RNA Interference (RNAi) is one of the most exciting discoveries of the past decade in functional genomics. RNA interference is the biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by targeting mRNA, resulting in a reduction in the expression of a particular gene in mammalian cell systems (Nencioni et al., 2004). It is clear that RNAi has rapidly become an important research tool for gene silencing. Long double-stranded RNAs (dsRNAs) can be used to silence the expression of target genes in a variety of organisms and cell types (Zhang et al., 2004). Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. The dsRNAs are processed by an RNase-like enzyme (Dicer) into small interfering RNAs (siRNAs). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). Activated RISCs subsequently bind to complementary transcripts by base pairing interactions and cause gene silencing (Figure 2.5).

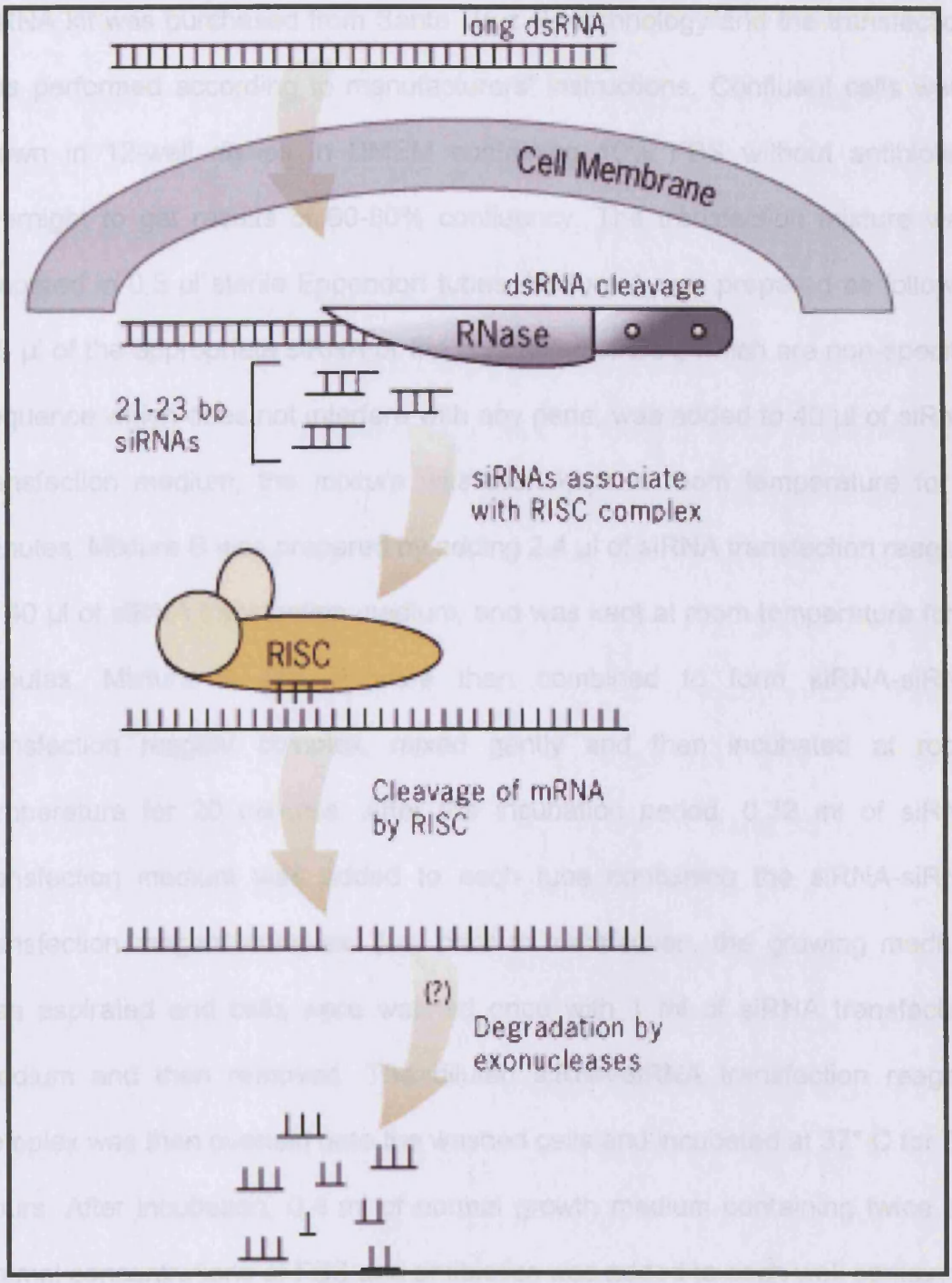



**Figure 2.5: Overview of RNA Interference**

Upon introduction of the dsRNAs into the cell, they are processed by an RNase-like enzyme (Dicer) into small interfering RNAs (siRNAs). The siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). Activated RISCs subsequently bind to the transcripts and cause cleavage of mRNA leading to gene silencing. Adapted from www.sirna.com

SiRNA kit was purchased from Santa Cruz Biotechnology and the transfection was performed according to manufacturers' instructions. Confluent cells were grown in 12-well dishes in DMEM containing 10% FBS without antibiotics overnight to get results of 60-80% confluency. The transfection mixture was prepared in 0.5 µl sterile Eppendorf tubes. Mixture A was prepared as follows: 3.6 µl of the appropriate siRNA or the scrambled siRNA, which are non-specific sequence which does not interfere with any gene, was added to 40 µl of siRNA transfection medium, the mixture was then kept at room temperature for 5 minutes. Mixture B was prepared by adding 2.4 µl of siRNA transfection reagent to 40 µl of siRNA transfection medium, and was kept at room temperature for 5 minutes. Mixture A and B were then combined to form siRNA-siRNA transfection reagent complex, mixed gently and then incubated at room temperature for 20 minutes. After the incubation period, 0.32 ml of siRNA transfection medium was added to each tube containing the siRNA-siRNA transfection reagent complex. Just prior to transfection, the growing medium was aspirated and cells were washed once with 1 ml of siRNA transfection medium and then removed. The diluted siRNA-siRNA transfection reagent complex was then overlaid onto the washed cells and incubated at 37° C for 5-7 hours. After incubation, 0.4 ml of normal growth medium containing twice the normal concentrations of FBS and antibiotics was added to each well containing transfected cells without removing the transfection mixture and incubated for an additional 24 hours. Once incubation was complete, transfection medium was replaced with fresh normal growth medium and the appropriate reagent was then added to incubate for the appropriate time point. Cells were then collected for Western blotting, other biochemical assay and ELISA.

### 2.5.5 Western Blot Analysis

Total protein of each sample was calculated as described in section 2.4.1. Appropriate volumes of each sample were combined with loading buffer (Laemmli buffer, obtained from Bio-Rad Laboratories Ltd., containing 5% (v/v) β-mercaptoethanol) to a give a total volume 30 µl, so that the final concentration loaded onto each lane was equal to 30 µg. The HO-1 positive control (Bio-Quote Ltd., York, UK) was diluted to 1 µg/ml in loading buffer. Samples were pulsed for 20-30 sec on a bench-top microcentrifuge (MSE Microcentaur, Sanyo Gallenkamp Plc., Leicestershire, UK) followed by denaturing of the proteins for 10 min at 90oC on a heating block (Techne DB2A, Techne GmBH, Germany). The molecular weight marker (MWM) (Invitrogen LifeTechnologies Ltd.) was not heated. After heating, the samples were re-pulsed and 30 µl of samples, positive control, and MWM (10 µl) was loaded carefully using gel loading tips (Fischer, UK) into the wells of a (12% tris-glycine Ready gel for HO-1, Nrf2 and NF-KB, or 7.5% for iNOS protein analysis) (Bio-Rad Laboratories Ltd.). Electrophoresis was carried out at room temperature in a tank containing running buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS) (Fischer, UK) using the Mini-PROTEAN® II system and Power-Pac 300 power supply (Bio-Rad Laboratories Ltd.) at a constant voltage of 125 V until the loading buffer had migrated to, but not beyond the base of the gel. The subsequent procedures were carried out using the Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories Ltd.). While the gel was running appropriately sized sheets of nitrocellulose membrane (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and 3MM Whatman™ blotting paper were cut and pre-soaked in dH2O and cold (4oC) transfer buffer (0.025 M Tris, 0.192 M

glycine, 0.1% (v/v) SDS, 20% (v/v) methanol) for 10 and 5 min, respectively. After electrophoresis was completed a 'gel sandwich' (Figure 2.6) was prepared upon the clear (positive electrode) side of a gel cassette. After removing any air bubbles the cassette was closed securely via the latch and placed into the electrode module with the black (negative electrode) side facing the black panel of the module. This orientation ensures the proteins migrate from the gel onto the nitrocellulose membrane and not into the transfer buffer while transferring. Overnight transfer was carried out at 4°C with a constant voltage of 30 V using a transfer unit (Bio-Rad Laboratories Ltd).

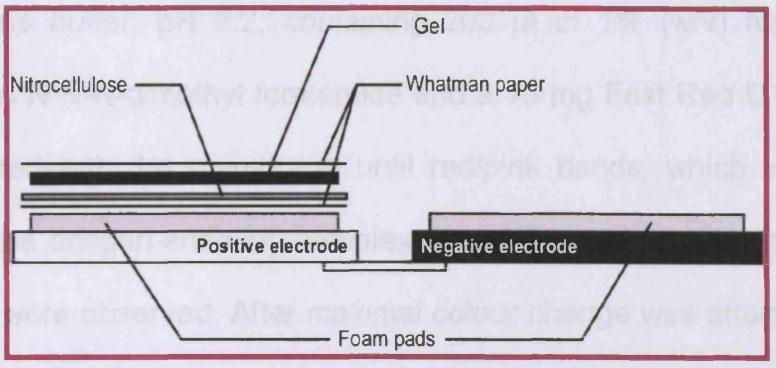



**Figure 2.6: Arrangement of gel sandwich assembly**

All subsequent procedures were performed at room temperature on an orbital shaker (Denley Instruments Ltd., Sussex, UK) at low speed. Non-specific binding of the antibodies was prevented by incubating the membrane in blocking solution (5% (w/v) non-fat dried milk in PBS (0.01 M phosphate buffer, pH 7.4, containing 0.2% Tween-20) for 2 h at room temperature, followed by a single 5 min wash with 10 ml of PBS (pH 7.4). The membrane was then incubated for 2 h with anti-HO-1 (Stressgen Biotechnologies Corporation, Victoria, BC, Canada) anti-Nrf2, or anti-NF-KB (Santa Cruz Biotechnology,

Santa Cruz, CA, USA) and β-actin (Abcam) primary antibodies, in tris buffered saline (TBS) (0.05 M Tris-HCl, 0.0037 M KCl, 0.7137 M NaOH, pH 7.4). Next, the membrane was washed three times, once with PBS-T (PBS 0.01 M, pH 7.4, containing 0.05% (v/v) Tween 20), then twice with TBS (pH 7.4). Proteins were visualised using an ExtrAvidin® alkaline phosphatase staining kit (Sigma). The biotinylated anti-rabbit IgG antibody, diluted 1:1000 in TBS (pH 7.4), was incubated with the membrane for 1 h. The membrane was washed three times with TBS (pH 7.4) and the ExtrAvidin® alkaline phosphatase conjugate, diluted 1:1000 in TBS (pH 7.4) applied for 1 h. The three washes with TBS (pH 7.4) were repeated. After the last wash, a freshly prepared substrate solution (9.8 ml of 0.1 M Tris buffer, pH 8.2, containing 200 µl of 1% (w/v) Napthol-AS-BI phosphate in N-N-N-dimethyl formamide and a 10 mg Fast Red DT salt tablet) was incubated with the membrane until red/pink bands, which indicates the location of the antigen-antibody complexes of the protein on the nitrocellulose membrane, were observed. After maximal colour change was attained (after 10 minutes) any excess substrate solution was washed off with $dH_2O$ and the membrane stored in foil, until scanned using photoshop software.

### 2.5.6 Isolation and Determination of Total HO-1 mRNA

In order to maintain the integrity of the ribonucleic acid (RNA) and prevent contamination or degradation by RNases (enzymes that degrade RNA) a number of precautions were taken: all glassware were sterilised and the equipment and solutions were washed and prepared with $dH_2O$ which was pre-treated with diethyl pyrocarbonate (DEPC). The latter was prepared by adding DEPC (0.5 ml/litre) to $dH_2O$, within pre-autoclaved glass bottles, shaking

vigorously to mix, followed by baking at 40°C for 24 h. The water was cleaned of any remaining DPEC by autoclaving at 121°C for 25 min.

After being treated, cells were harvested as mentioned in section 2.2.3. Cells were then resuspended in 1X PBS, transferred into 1.5 ml Eppendorf tubes, and kept in the -80°C freezer until being used for RNA extraction. RNA isolation was performed using Purescript® RNA Isolation Kit (Gentra, Minneapolis, MN 55441 USA). RNA isolation was performed according to the manufacturers' instructions. Briefly, cells were centrifuged at 13,000-16,000 g for 5 seconds; the supernatant was removed with a pipette leaving behind a visible cell pellet and 10-20 µl of residual liquid. The pellets were then vortexed vigorously to resuspend the cells in the residual supernatant, 300 µl of the cell lysis solution was added, and pippetted up and down for 3 times to lyse the cells. 100 µl of the protein precipitation solution was then added to the cell lysates, the tubes containing the cell lysate were gently inverted 10 times and then placed into ice for 5 minutes before being centrifuged at 13,000 g for 3 minutes. The supernatant (containing the RNA) was poured into a clean 1.5 ml Eppendorf tube containing 300 µl 100% propan-2-ol. The samples were then gently inverted 50 times, after which they were centrifuged at 13,000 g for 3 minutes; the supernatant was discarded, leaving the RNA as a small pellet. The pellets were then washed using 300 µl of 70% ethanol, the samples were centrifuged at 13,000 g for 1 minute and the ethanol was poured off gently. The tubes containing the RNA pellets were then inverted to drain and air dry for 30 minutes and the RNA was resuspended with15 µl of RNA hydration solution, samples were then kept in the -80°C freezer until needed.

The RNA concentration was determined spectrophotometrically with 3 µl of each sample diluted in 97 µl of DEPC water. The absorbance was read at 260 nm and 280 nm against a blank of DPEC water using a UV-visual spectrophotometer (Helios α, Unicam Ltd., Cambridge, UK). The total RNA concentration of the entire sample was derived from Equation 2-3. The quality of the extracted RNA was based on the ratio of the readings at $OD_{260}/OD_{280}$. A ratio of 1.6-1.8 indicates good quality while anything less that 1.6 signifies that the sample contains DNA instead of RNA.

$$OD_{260} \times 4 = RNA(\mu g / \mu l)$$

**Equation 2-3 Calculation of total RNA concentration**

### 2.5.7 Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis of HO-1 mRNA

Having isolated and calculated the total RNA concentration as described above, sufficient DPEC water was added to each sample so that a final concentration of 500 ng RNA was obtained. The RT reaction was carried out in a 50 µl reaction volume using Ready-To-Go™ RT-PCR Beads (Amersham Biosciences UK Ltd., UK) in 0.2 ml tubes. Each bead contains all the reagents necessary for the reaction (i.e. Moloney murine leukaemia virus (M-MuLV) RT in 1.5 mM $MgCl_2$, *Taq* DNA polymerase and RNase inhibitor) except primers. To each reaction tube 35 µl of DPEC water was added and left for 5-10 min to dissolve the beads. Next, 5 µl of first strand primer (pd $(N)_6$ random hexamer primers) and 2 µl of sample was added to each tube and mixed by gentle pipetting. A 50

µl aliquot of mineral oil was added onto the latter mixture to prevent exposure to air and RNAase. Then the tubes were placed in a UNO II thermal cycler (Biometra Ltd., Maidstone, Kent, UK) and underwent RT denaturation for a duration of 20 min (15 min at 42 °C and 5 min at 95°C). The RT reaction was amplified by PCR, for this 8 µl of primer mixture (2 µl) of the primers ( ).

**Table 2-2: the sequence of the primers**

| | |
|---|---|
| HO-1 sense | AGTATCCTTGTTGACACGGC |
| HO-1 antisense | GTTAGACCAAGGCCACAGTG |
| GAPDH sense | GGAGTCAACGGATTTGGT |
| GAPDH antisense | GTGATGGGATTTCCATTG |

The primers were placed in 152 µl of DPEC water added under the mineral oil, the second cycle parameters consisted of a 30 sec denaturing step at 94°C, a 30 sec annealing step at 60°C and a 1 h 30 min extension step at 68°C. A total of 26 cycles were used per amplification and the annealing temperature for HO-1 and GAPDH was 65°C. In the mean time a 1.5% agarose gel (1.5 g of agarose in 100 ml of 0.5% TBE buffer (10 mM Tris-HCl, 1 mM EDTA), containing 0.5 µg/ml of ethidium bromide (EB) was prepared. The gel was poured into a gel casting mould and pre-run for 10 min at 120 V in a tank containing 0.5% TBE buffer. After completion of the PCR the samples were placed on ice while prepared for running on the gel. To a fresh set of Eppendorf

tubes 3 µl of loading buffer and 10 µl of sample (taken from below the mineral oil layer) was added and mixed by gentle pipetting. A molecular weight marker (5 µl of 100 base pair PCR ladder, 5 µl of DPEC water and 3 µl of loading buffer) was prepared in parallel. Aliquots (13 µl) of samples and molecular weight marker were carefully loaded into their respective wells, their loading order recorded, and the gel was run at 120 V for about 1-1.5 h. The PCR products were visualised using a UV dual-intensity transilluminator TM 20 (UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, UK) and photographed using a Polaroid DS 34 camera with a DS H-8 hood (Polaroid UK Ltd., St Albans, Hertfordshire, UK) and Polaroid film (Type 667) with a No. 15, deep yellow filter.

## 2.6 Statistical Analysis

Statistical analysis was performed using one-way analysis of varience (ANOVA) combined with Bonferroni test. Data were presented as mean ± SEM and differences were considered significant at $P < 0.05$. All calculations were performed using a commercially available statistical package (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

# 3 THE SEARCH FOR INDUCERS OF HO-1 AMONGST PHYTOCHEMICALS

## 3.1 Introduction

Diets rich in fruit and vegetables are associated with lower risks of developing various diseases-mediated by oxidative stress, such as atherosclerosis (Talalay and Fahey, 2001). Several mechanisms are involved in the protective effects of fruit and vegetables, which undoubtedly include the endogenous cytoprotective mechanisms, such as endogenous detoxifying enzymes, i.e. phase II enzymes (Talalay and Fahey, 2001). Phase II enzymes protect against damage by electrophiles and therefore emerging as a very important component of cellular defenses against oxidants (Talalay, 2005). Indeed, ample studies support the view that induction of phase II enzymes is an important protective mechanism against ROS (Moskaug et al., 2005). It is therefore of great importance to search for different pharmacological agents, which possess intrinsic abilities to modulate the expression of these protective enzymes (Balogun et al., 2003b). Dietary phytochemicals are emerging as novel modulators of cellular functions, because of their intrinsic ability to selectively and potently induce phase II enzymes (Moskaug et al., 2005) namely, HO-1 (Xu et al., 2005). The induction of many antioxidants and phase II drug-metabolizing enzymes by phenolic antioxidants and electrophilic compounds is regulated at the transcriptional level (Talalay and Fahey, 2001). It has been demonstrated that MAPK are involved in the activation of the HO-1 expression by some phenolic compounds, for example, p38 pathway was involved in the mechanisms of HO-1 gene induction by quercetin in rat aortic smooth muscle cells (Lin et al., 2004), by carnosol in

PC12 cells (Martin et al., 2004) and by curcumin in renal epithelial cells (Balogun et al., 2003b). In this chapter, we investigated a number of polyphenolic compounds, namely, phloretin, which is abundantly present in apples (Tsao et al., 2003) and possesses anti-oxidative properties, and accounts in part for the anti-oxidative capacity of apples (Lee et al., 2003). Another compound which belongs to the family of phytochemicals is baiclein, which has potent cytoprotective properties, including anti-oxidant properties; it prevented endothelial dysfunction in spontaneously hypertensive rat aorta (Machha and Mustafa, 2005) and prevented lipid peroxidation in mice (Im et al., 2005). Epigallocatechin 3-O-gallate (EGCG) is one of the major polyphenols in green tea (Nakai et al., 2005), and was shown to be protective against oxidation-induced damage in erythrocytes (Rizvi et al., 2005). Another exciting group is the chalcones, which are emerging as potent modulators of endogenous cytoprotective enzymes (Alcaraz et al., 2004). Chalcones are a group of phenolic compounds which belong to the flavonoids family, and widely occur in nature as pigments, they are present in hops plants and liquorice (Ivanov and Ronai, 2000). Chalcones have been reported to possess a variety of beneficial biological properties (Anto et al., 1995) (Ban et al., 2004), including anti-inflammatory (Alcaraz et al., 2004), antioxidant and anti-bacterial activities (Haraguchi et al., 1998) (Yu et al., 1995). In the present study, we are interested in a particular derivative of chalcones, i.e. 2′-hydroxychalchone (2-HC) which exerts its cytoprotective actions through the activation of specific transcriptional factors, such as Nrf2 (Alcaraz et al., 2004) and by up-regulation of endogenous cytoprotective pathways (Foresti et al., 2005). It has been demonstrated that

chalcones possess potent anti-oxidant properties; chalcones protected BAEC from $H_2O_2$-induced cellular damage (Foresti et al., 2005).

## 3.2 Objectives

To investigate if baiclein, phloretin, EGCG and 2-HC have the ability to induce haem oxygenase activity and HO-1 expression in BAEC and to elucidate the cellular mechanisms involved in the induction of HO-1.

## 3.3 Materials and Methods

### 3.3.1 Reagents

2-HC was purchased from INDOFINE chemical company INC (Belle Mead, NJ 08502). All other reagents were purchased from Sigma unless otherwise specified.

### 3.3.2 Cell Culture

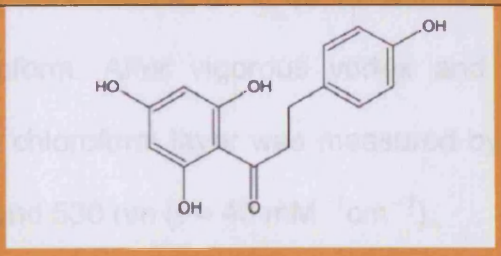
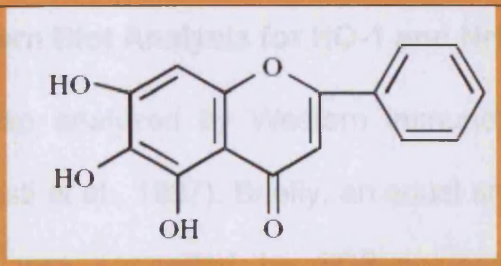
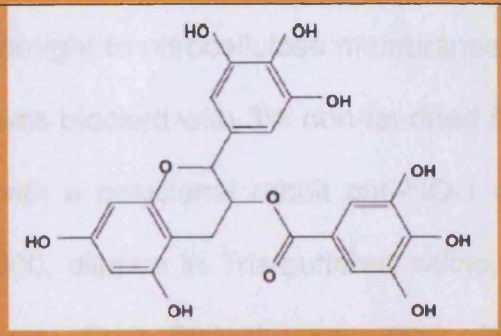
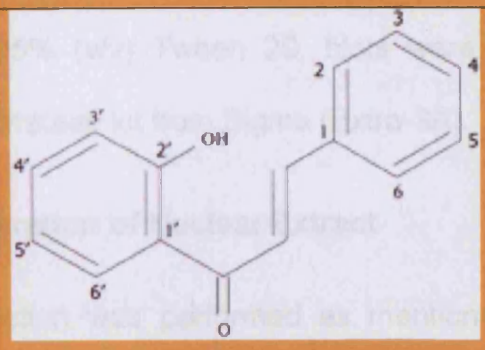
Bovine Aortic Endothelial cells (BAEC) were purchased from Coriell Cell Repositories (Camden, NJ, U.S.A.) and cultured in ISCOVES' medium which contains: 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and supplemented with 10% FBS. Cells were grown in 75-$cm^2$ flasks and maintained at 37°C in a humidified atmosphere of air and 5% $CO_2$.

### 3.3.3 Experimental Protocol

BAEC were exposed to various concentrations (5, 15 and 30 μM) of polyphenolic compounds phloretin, baiclein, and EGCG (Table 3-1) for 6 h, haem oxygenase activity was then determined. Haem oxygenase activity and HO-1 protein expression were also measured in cells exposed for 6, 18, and 36 h to 2-HC (Table 3-1). Cell viability was assessed using a metabolic assay (Alamar Blue) and LDH release after 24 and 36 h-incubation. To assess the potential anti-oxidant effects of 2-HC, cells were pretreated with 2-HC for 6 h followed by a 2-h exposure to various concentrations (0.75, 1.5 and 3 mM) of $H_2O_2$. The participation of the MAPK pathway in the increase of haem oxygenase activity by 2-HC was assessed using commercially avalailbe inhibtors of the three pathways; PD098059 (ERK inhibitor, 25 μM), SB203580 (p38 inhibitor, 5 μM) or SP600125 (JNK inhibitor, 10 μM). An inhibitor of the PI3K pathway (LY294002, 25

μM) was also tested. Furthermore, in order to investigate the effect of 2-HC on the translocation of the transcription factor Nrf2 to the nucleus, cells were pre-incubated with 2-HC for 30 and 60 minutes. At the end of the experimental protocol, nuclear extraction was performed as mentioned previously.

**Table 3-1: The chemical structure of baiclein, phloretin, EGCG and 2-HC**

| Compound | Chemical structure | Cytoprotective properties | Ref. |
|---|---|---|---|
| **Phloretin** |  | Anti-oxidant | [147] |
| **Baiclein** |  | Anti-oxidant, | [114;157] |
| EGCG |  | Anti-oxidant, anti-inflammatory | [68;233] |
| **2-HC** |  | Anti-oxidant, anti-inflammatory and anti-tumour | [165] |

### 3.3.4 Haem Oxygenase Activity Assay

Haem oxygenase activity was determined at the end of each treatment as described previously by our group (Foresti et al., 1997) (Motterlini et al., 2000a). Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat liver cytosol as a source of biliverdin reductase, and the substrate haemin. The reaction mixture was incubated in the dark at 37°C for 1 h and was terminated by the addition of 1 ml of chloroform. After vigorous vortex and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm ($\varepsilon$ = 40 $mM^{-1}cm^{-1}$).

### 3.3.5 Western Blot Analysis for HO-1 and Nrf2

Cells were also analyzed by Western immunoblot technique as previously reported (Foresti et al., 1997). Briefly, an equal amount of proteins (30 µg) from each sample was separated by SDS–polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes, and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1000, dilution in Tris-buffered saline, pH 7.4) Nrf2 (1:500 dilution) antibodies (Santa Cruz Biotechnology Inc.). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A).

### 3.3.6 Preparation of Nuclear Extract

Nuclear extraction was performed as mentioned previously (Balogun et al., 2003b). Briefly, cells were washed twice with cold 1X PBS and harvested by

centrifugation at 800 g for 3 minutes at 4°C. Cells were then carefully resuspended in a cold buffer (buffer A) which contains 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1mM EDTA, and 0.1 EGTA, 10% NP-40 and protease inhibitor cocktail (Roche) and incubated on ice for 15 minutes. The homogenate was then centrifuged at 800 g for 3 minutes; the pellet was then resuspended and incubated for 15 minutes with buffer B, which contains 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, and 1 mM EGTA. Finally the samples were spun at 15,000 g for 5 minutes and the supernatant was kept in the -80 freezer until needed.

### 3.3.7 Cell Viability/Alamar Blue Assay

Cell viability was determined using an Alamar Blue assay kit, it was carried out according to the manufacturer's instructions (Serotec, U.K.) as previously reported (Motterlini et al., 2000b). The assay is based on the detection of metabolic activity of living cells using a redox indicator, which changes from an oxidized (blue) form to a reduced (red) form. The intensity of the red color is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 and 600 nm and expressed as a percentage of control.

### 3.3.8 LDH Assay

Extracellular, i.e., released, lactate dehydrogenase (LDH) activity was measured using cytotoxicity detection kit (Roche). At the end of the period of incubation, cell supernatant was collected, and then any cell residue was removed by centrifugation at (250xg). The reaction mixture (which is composed of the catalyst and the dye solution) was then added to the cell-free

supernatant, incubated for 15 minutes, and then the absorbance was measured at 490 and 600 nm. Cellular LDH activity was determined after lysis of the cells with Triton X-100 (1% in DMEM at 25°C). Released LDH was expressed as percentage of total LDH activity.

### 3.4 Statistical Analysis

Differences among the groups were analyzed using one way ANOVA .Values were expressed as mean ± S.E.M., and differences between groups were considered to be significant at $p < 0.05$.

## 3.5 Results

### 3.5.1 The Effect of Baiclein, Phloretin and EGCG on Haem Oxygenase Activity in Bovine Aortic Endothelial Cells

We wanted to examine whether baiclein, phloretin and EGCG have the ability to induce haem oxygenase activity in BAEC, for this purpose, cells were incubated with various concentrations (5-30 μM) of each of the compounds. As illustrated in Figure 3.1, exposure of cells for 6 h to various concentrations of baiclein (Figure 3.1 A), phloretin (Figure 3.1 B), or EGCG (Figure 3.1 C) did not have any significant effects on haem oxygenase activity.

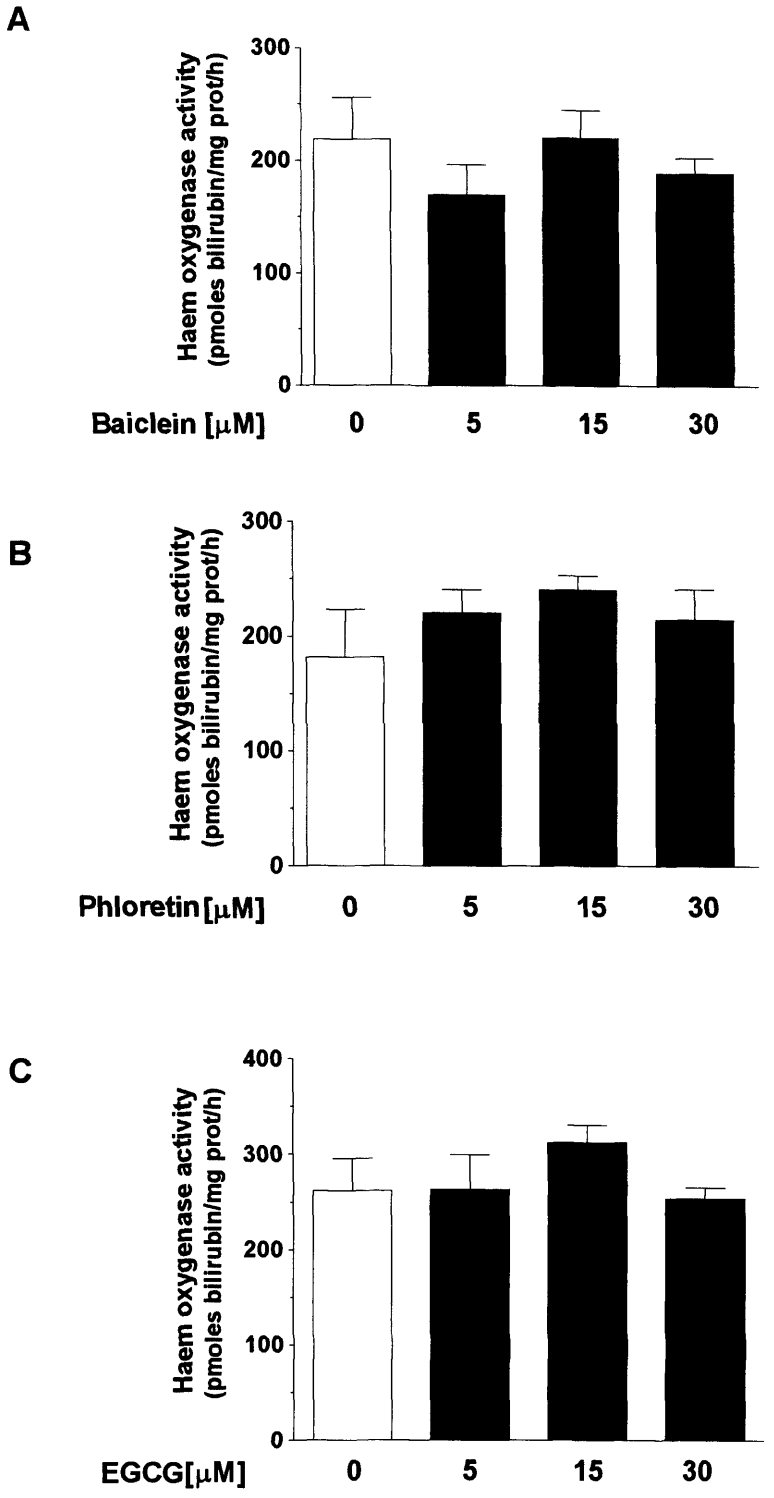



**Figure 3.1: The effect of baiclein, phloretin and EGCG on haem oxygenase activity in BAEC**

BAEC were exposed to various concentrations (5-30 μM) of baiclein(A) phloretin (B) and EGCG (C) for 6 h. Haem oxygenase activity was then determined at the end of the incubation. Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 6 independent experiments per group.

### 3.5.2 2-HC Induces Haem Oxygenase Activity and HO-1 Expression in BAEC

In order to establish the effect of 2-HC on haem oxygenase activity and HO-1 protein expression, we measured the haem oxygenase activity and HO-1 protein expression in BAEC exposed to 2-HC for three different time points. As shown in (Figure 3.2), exposure of BAEC to various concentrations (5, 15 and 30 μM) of 2-HC, resulted in a concentration and time-dependent increase in haem oxygenase activity and HO-1 protein expression; at 6 h (Figure 3.2 A), 18 h (Figure 3.2 B), however, after 36 h-incubation, there was a significant decrease in haem oxygenase activity and HO-1 protein expression (Figure 3.2 C).

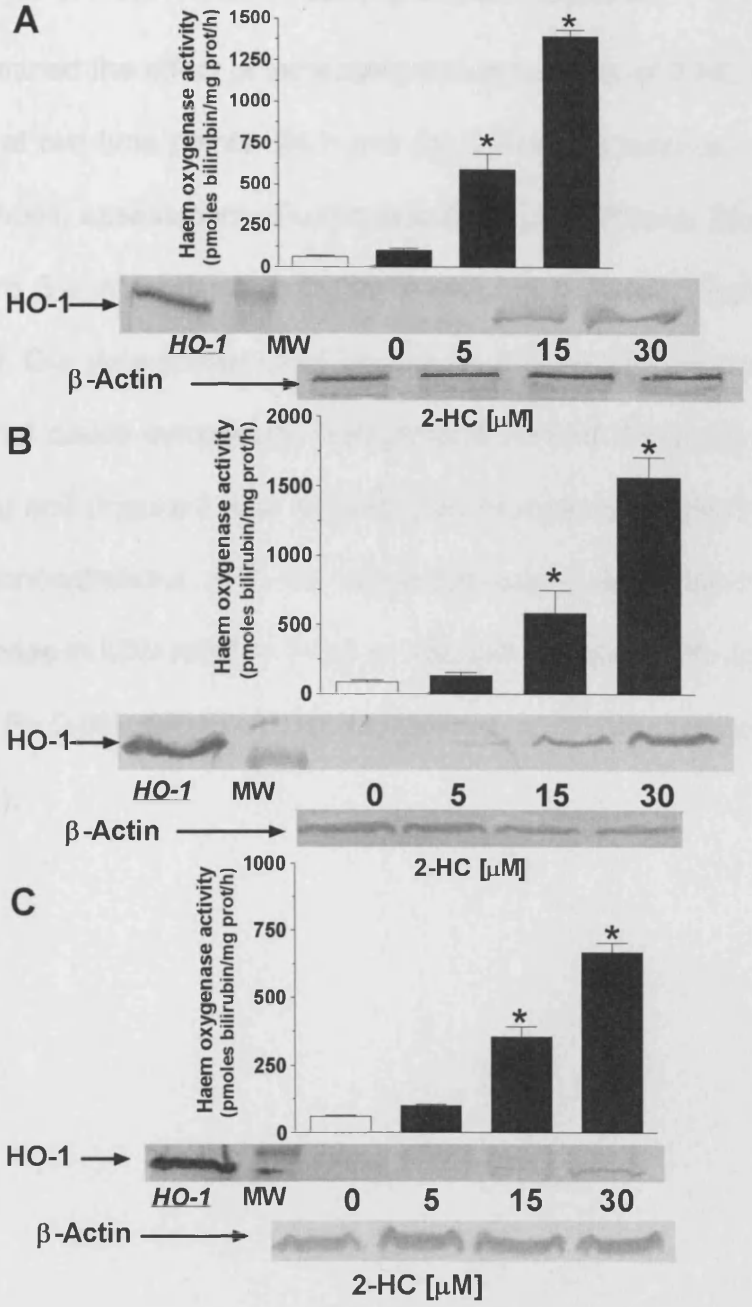



**Figure 3.2: The effect of 2-HC on haem oxygenase activity and HO-1 expression in BAEC**

BAEC were exposed to various concentrations (5-30 μM) of 2-HC for 6 h (A) 18 h (B) and 36 h (C). Haem oxygenase activity and HO-1 protein expression were determined at the end of the incubation. Cells treated with medium alone represented control (0). Each Western Blot is a representative of three independent experiments. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. control. *HO-1:* positive control recombinant HO-1 protein, MW: molecular weight marker.

### 3.5.3 Effects of 2-HC on Cell Viability and Metabolism

We next examined the effect of increasing concentrations of 2-HC (5-30 μM) on cell viability, at two time points, 24 h and 36 h. For this purpose, we used two different methods, assessment of cell metabolism using Alamar Blue (Figure 3.3 A, and Figure 3.4 A) and measurements of LDH release (Figure 3.3 B and Figure 3.4 B). Our data showed that incubation of BAEC for 24 h with 2-HC (5-30 μM) did not cause cytotoxicity compared to control (Figure 3.3). However, (Figure 3.4 A) and (Figure 3.4 B) showed that incubation of BAEC for 36 h with increasing concentrations of 2-HC demonstrated a slight (but insignificant, $P>0.05$) increase in LDH release (5%) at 5-30 μM (Figure 3.4 B) and a mild (but insignificant, $P> 0.05$) decline in cell metabolism at 30 μM compared to control (Figure 3.4 A).

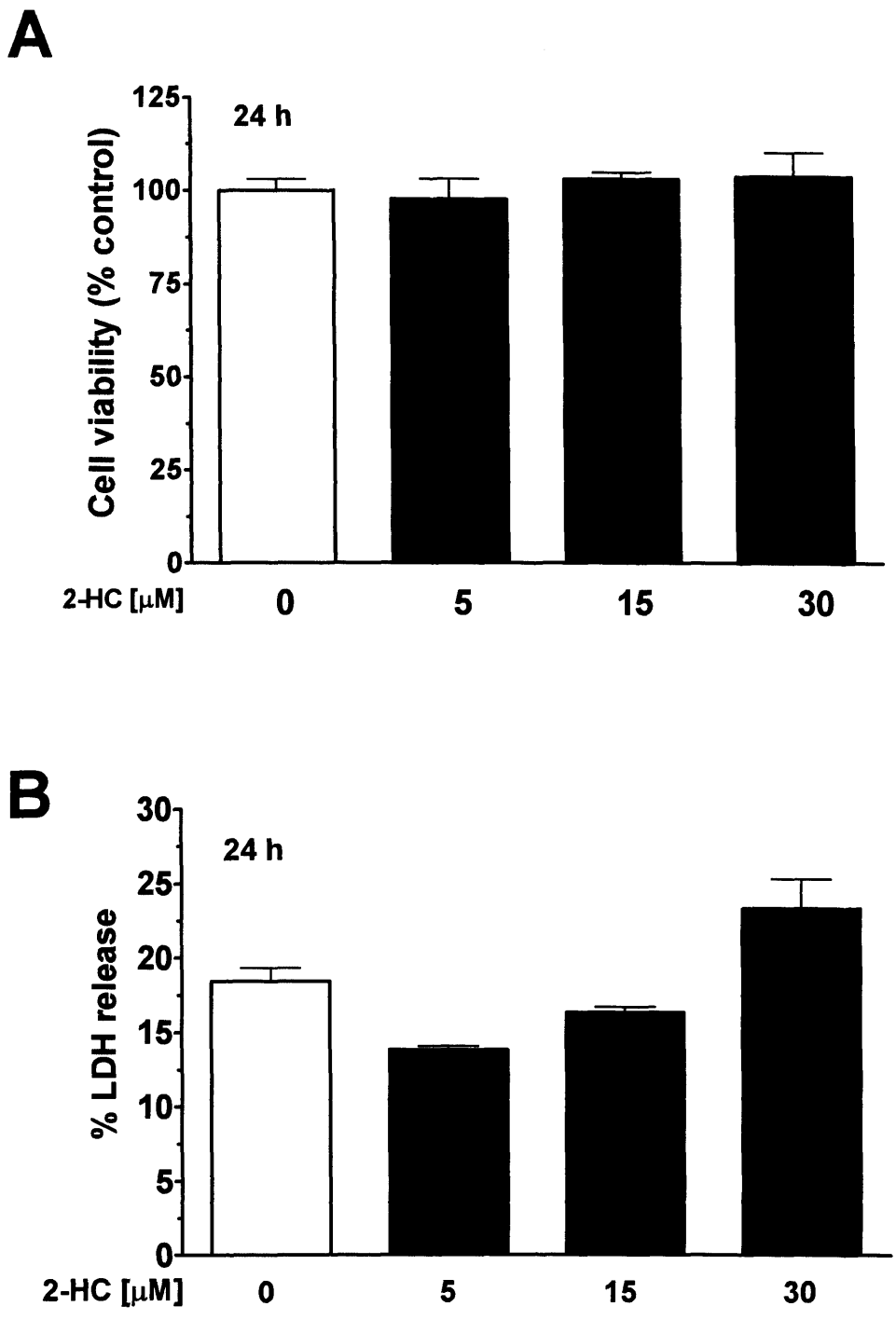



**Figure 3.3: Viability of cells exposed to 2-HC after 24 h incubation.**

BAEC were exposed for 24 h in the presence of 2-HC (5-30 μM) and the viability was then determined using Alamar Blue (A). Cells were exposed to 2-HC (5-30 μM) for 24 h and damage was assessed by measuring LDH release (B). Bars represent the mean ± S.E.M. of 5-6 independent experiments.

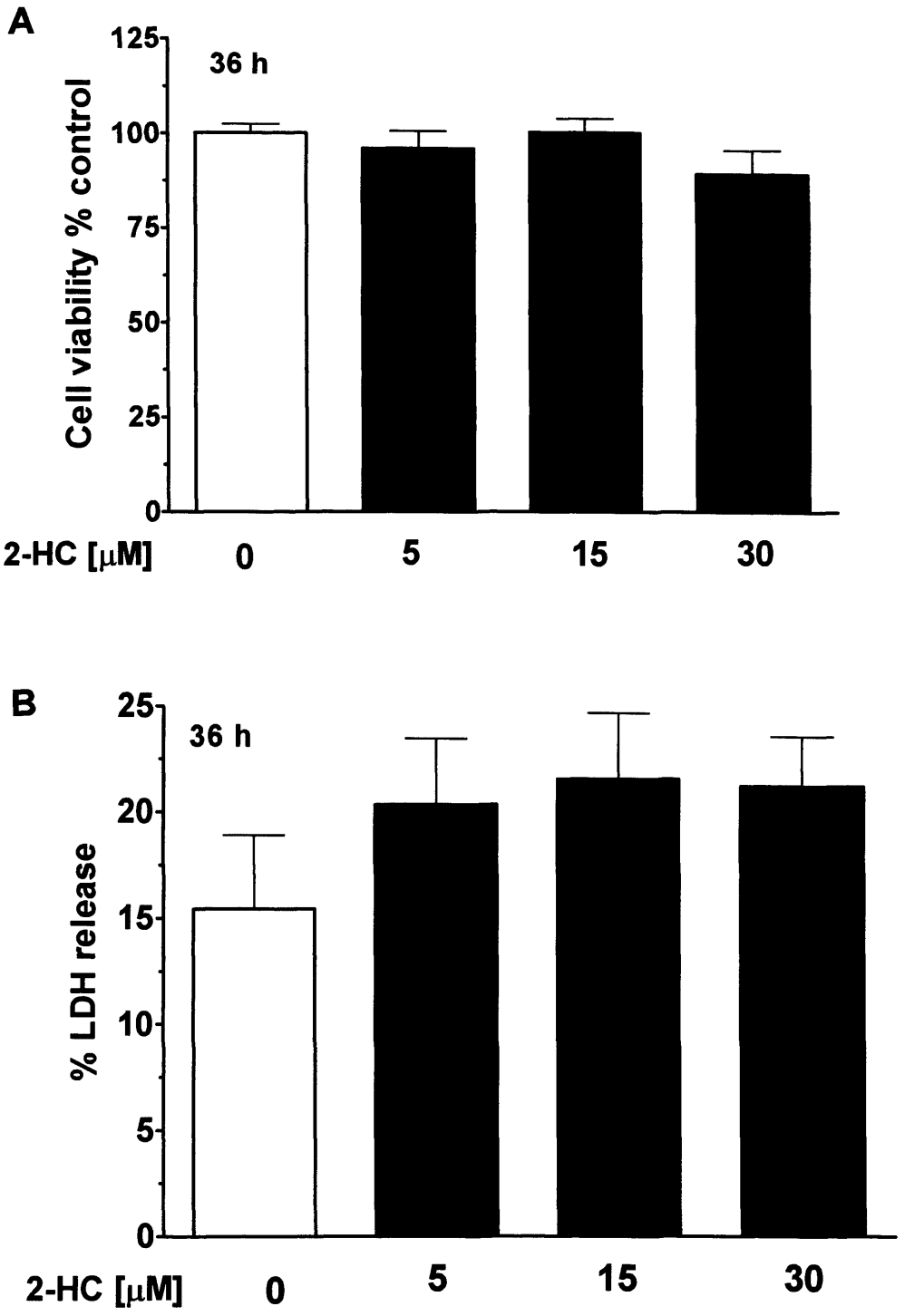



**Figure 3.4: Viability of cells exposed to 2-HC after 36 h incubation**

BAEC were exposed for 36 h in the presence of 2-HC (5-30 μM) and the viability was then determined using Alamar Blue (A). Cells were exposed to 2-HC (5-30 μM) for 36 h and damage was assessed by measuring LDH release (B). Bars represent the mean ± S.E.M. of 5-6 independent experiments.

### 3.5.4 2-HC Attenuates $H_2O_2$–Mediated Oxidative Stress

Having established that 2-HC is a potent inducer of hame oxygenase activity and HO-1 expression, we then tested if 2-HC could counteract the oxidative stress caused by treatment with $H_2O_2$. Cells were initially pretreated with 2-HC (5-30 μM) for 6 h to allow HO-1 induction to take place. Treatment with increasing concentrations of $H_2O_2$ (0.75, 1.5, and 3 mM) for 2 h resulted in a concentration-dependent decline in cell metabolism (50, 40 and 35% respectively) compared to control group, pre-treatment of cells with 2-HC (5 μM) did not have any significant effect on $H_2O_2$-mediated decrease in cell metabolism (Figure 3.5 A). However, at higher concentrations (15 and 30 μM), 2-HC significantly attenuated the $H_2O_2$-mediated cytotoxicity evident by a significant increase ($P<0.05$) in cell metabolism in a concentration-dependent manner, (Figure 3.5 B and C).

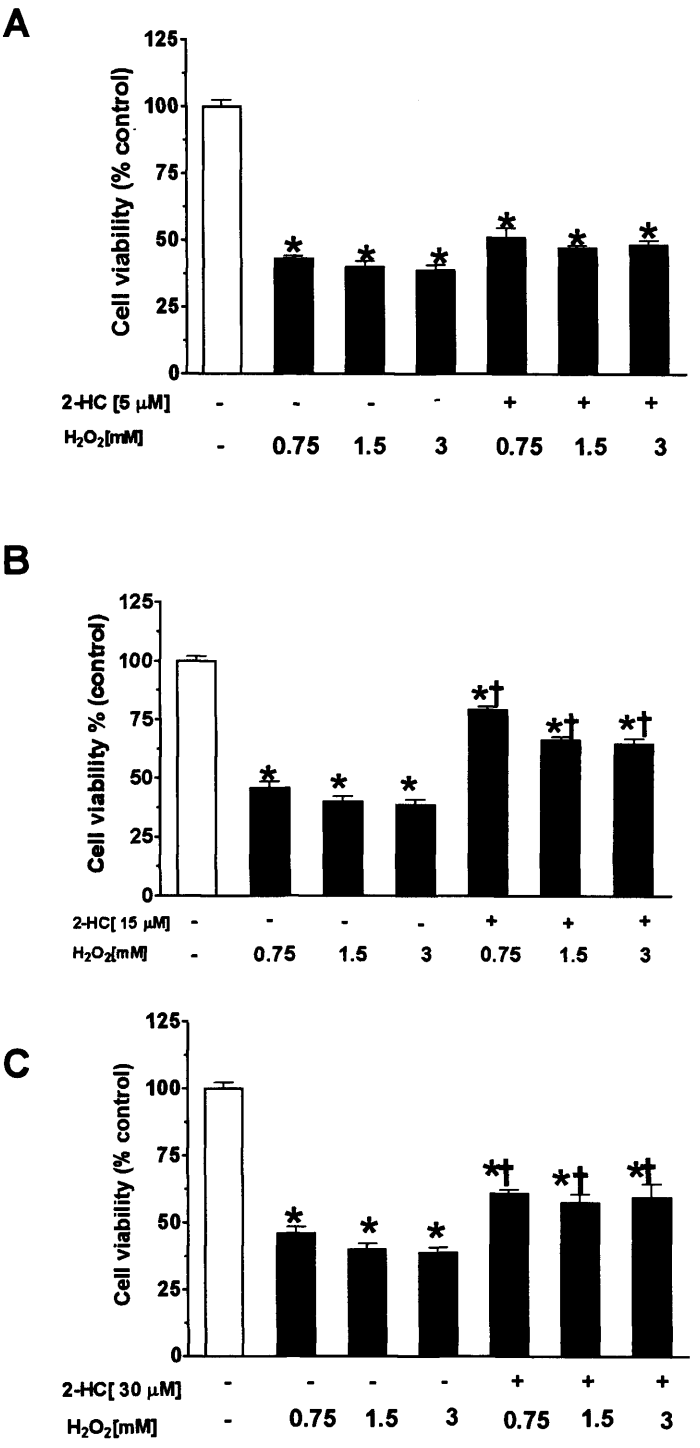



**Figure 3.5: Effect of 2-HC the $H_2O_2$–mediated oxidative stress**

Cells were pre-treated with of 2-HC 5 μM (A),15 μM (B) and 30 μM (C) for 6 h and then exposed to different concentrations of $H_2O_2$ for 2 h. Viability was then determined using Alamar Blue assay. Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control. † $< 0.05$ vs. $H_2O_2$.

### 3.5.5 The Effect of Actinomycin–D on 2-HC-Mediated Induction of Haem Oxygenase Activity

In order to investigate the mechanisms involved in 2-HC-mediated induction of haem oxygenase activity. Actinomycin–D, a universal inhibitor of gene transcription (Hill-Kapturczak et al., 2001) was used. Cells were pre-treated with actinomycin–D for 30 minutes followed by 2-HC (15 μM) for 6 hours. The presence of actinomycin-D totally inhibited the 2-HC-mediated increase in the activity of haem oxygenase (Figure 3.6) suggesting that the induction of haem oxygenase activity by this chalcone occurs at the transcriptional level.

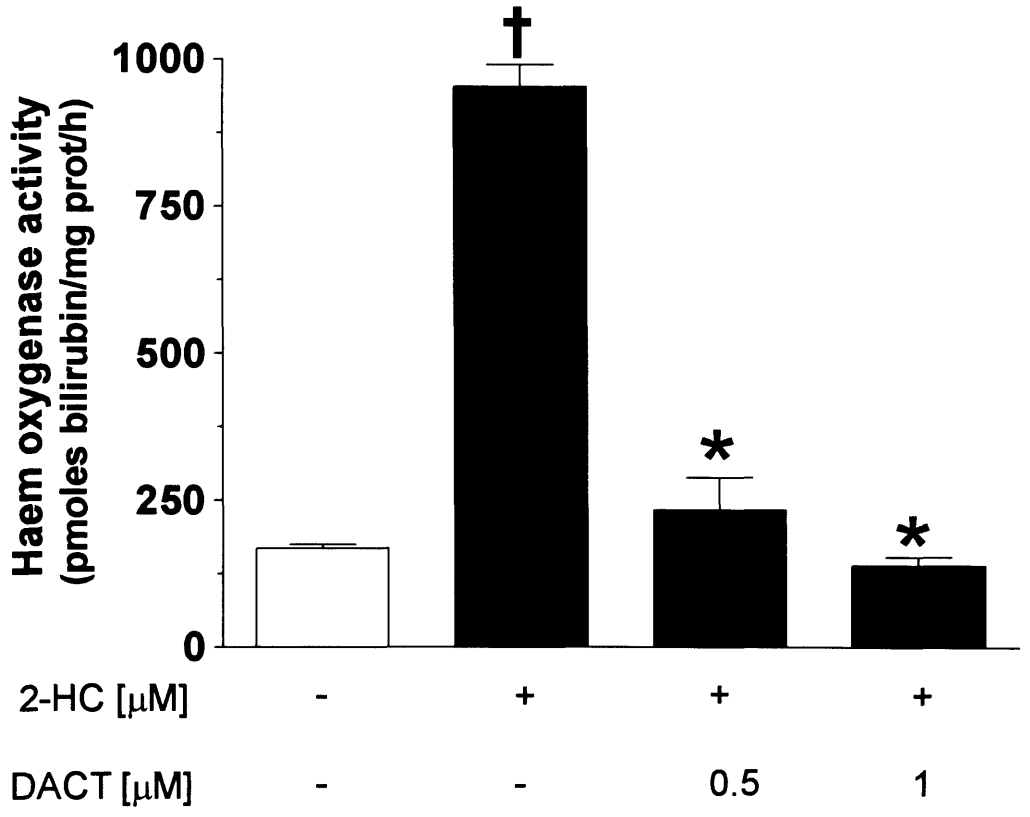



**Figure 3.6: The effect of actinomycin-D on the 2-HC-mediated stimulation of haem oxygenase activity in BAEC**

BAEC were pre-incubated with actinomycin-D for 30 min. then various concentrations (5-30 μM) of 2-HC for were added for 6 h and haem oxygenase activity was determined at the end of the incubation. Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 6 independent experiments per group.* $P < 0.001$ vs. 2-HC alone. † $P < 0.001$ vs control.

### 3.5.6 The PI3K Pathway is required For the Induction of Haem Oxygenase Activity and HO-1 Expression Mediated by 2-HC

To investigate the signalling cascade mediating the increase in haem oxygenase activity by 2-HC, we employed pharmacological antagonists of different MAPK family and PI3K pathways. As observed previously with endothelial cells (Foresti et al., 2005), inhibition of the ERK, JNK (Figure 3.7 A) and p38 (Figure 3.7 B) did not affect the induction of haem oxygenase activity by 2-HC. However, blockade of PI3K completely abolished the 2-HC-mediated up-regulation of haem oxygenase activity (Figure 3.7 B).

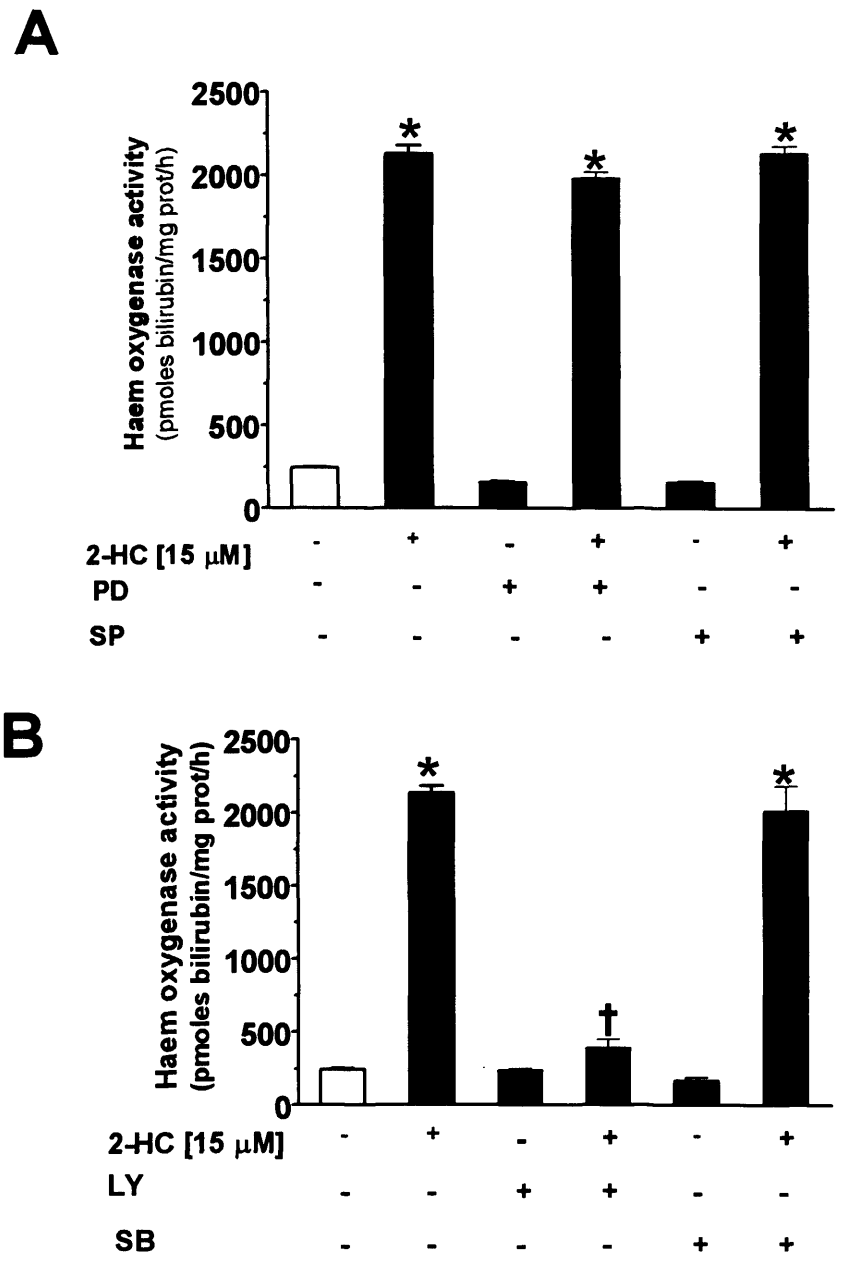



**Figure 3.7: Effect of MAPK inhibitors on the on the increase of haem oxygenase activity mediated by 2-HC.**

BAEC were pre-treated for 30 min. with PD 098,059 (ERK inhibitor, 25 μM) or SP 600125 (JNK inhibitor, 10 μM), in serum free-medium prior to exposure to 15 μM 2-HC for 6 h (A). Haem oxygenase activity was determined at the end of the incubation. BAEC were pre-treated for 30 min. LY 294002 (PI3K pathway inhibitor, 25 μM) or SB 203580 (p38 inhibitor, 5 μM) in serum free-medium prior to exposure to 15 μM 2-HC for 6 h (B). Haem oxygenase activity was determined at the end of the incubation. Bars represent the mean ± S.E.M. of 6 independent experiments, *P < 0.001 vs. Control, † represents p<0.001 vs. 2-HC alone.

### 3.5.7 Treatment with 2-HC Results in the Activation of Nrf2

Using Western blotting, we determined whether treatment of BAEC with 2-HC would result in the activation of Nrf2, i.e. the translocation of Nrf2 to the nucleus. For this purpose, cells were incubated with 2-HC for 30 min. As shown in (Figure 3.8), treatment with 2-HC resulted in the translocation of Nrf2 to the nucleus.

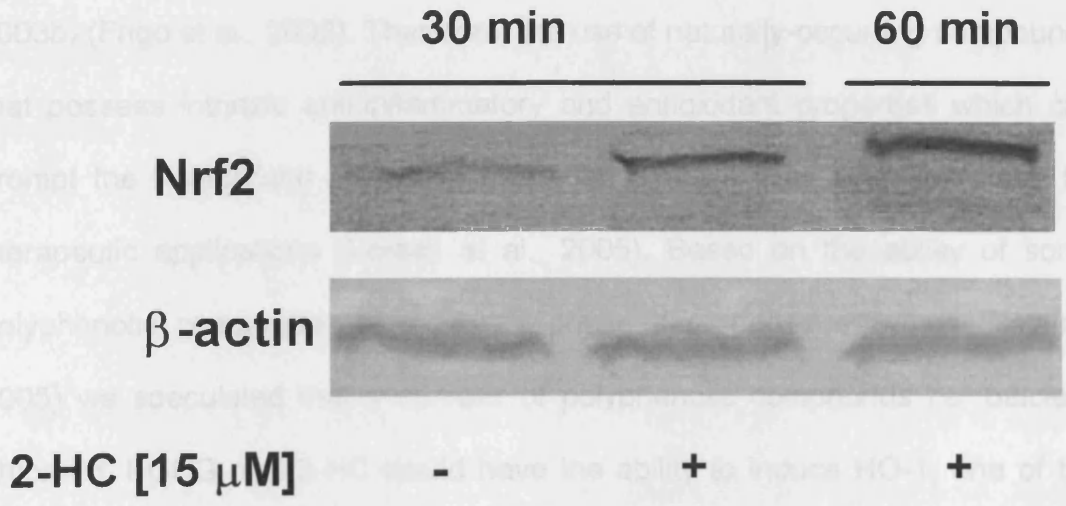



**Figure 3.8: Treatment with 2-HC results in the activation of Nfr2**

BAEC were pre-treated with 15 μM 2-HC for 30 min. and 60 min. The translocation of Nrf2 to the nucleus was determined in the nuclear extracts by Western Blot. Western Blot is a representative of three independent experiments. β-actin was used as an internal control for equal loading.

## 3.6 Discussion

Fruit and vegetables contain thousands of structurally diverse phytochemicals, of which a large fraction is polyphenols (Scalbert and Williamson, 2000). Furthermore, phytochemicals increase the efficacy of endogenous antioxidant defences and modulate the cellular redox state, a process which involves a wide range of signaling pathways and transcription factors (Balogun et al., 2003b) (Frigo et al., 2002). Therefore, the use of naturally-occurring compounds that possess intrinsic anti-inflammatory and antioxidant properties which can prompt the intracellular protective cascades offers a promising stratagem for therapeutic applications (Foresti et al., 2005). Based on the ability of some polyphenolic compounds to modulate phase II response enzymes (Talalay, 2005) we speculated that a number of polyphenolic compounds i.e. baiclein, phloretin, EGCG and 2-HC would have the ability to induce HO-1, one of the phase II response enzymes. Our data showed that baiclein, phloretin, EGCG did not induce haem oxygenase activity, however, treatment of cells with 2-HC induced haem oxygenase activity and HO-1 expression potently. Our results showed that the differential pattern of the ability to induce haem oxygenase activity amongst the four polyphenolic compounds tested could be explained on the basis of structural differences between these compounds. The presence of two structural elements was found to be required for high inducer potency: (i) hydroxyl groups at ortho-position on the aromatic rings and (ii) the beta-diketone functionality (Dinkova-Kostova and Talalay, 1999) (Dinkova-Kostova et al., 2001). This observation is supported by other studies in the HO-1 induction abilities of other phytochemicals (Scapagnini et al., 2002). However, based on our data, there is not enough evidence to support this view. Our results showed

that 2-HC is a potent inducer of haem oxygenase activity and HO-1 expression in BAEC, an effect which is observed after 6 and 18 h incubation, and to a lesser extent, after 36 h, the significant decrease in the haem oxygenase activity and the HO-1 expression after 36 h could be due to the fact that the compound have been metabolized by the cells, and it no longer presents in the medium to induce the expression of haem oxygenase activity. This is decidedly possible, because the decrease in the haem oxygenase activity and HO-1 expression is not due to cytotoxicity, because cells were still viable after 36 h-incubation with 2-HC. Our data is in agreement with data in the literature which recently documented that 2-HC and its derivatives are potent inducers of HO-1 expression, in endothelial cells (Foresti et al., 2005) and in RAW 264.7 murine macrophages (Alcaraz et al., 2004). Since the other 3 phytochemicals involved in this study (baiclein, phloretin and EGCG) did not have the ability to induce haem oxygenase activity, we did not study them further. Conversely, having established that 2-HC is a potent inducer of HO-1 expression, we wanted to study the mechanisms involved in the effect of 2-HC on HO-1, and examine the potential cytoprotective properties of this chalcone, in addition to the contribution of the HO-1 in these protective effects. Our data showed that actinomycin-D totally inhibited the 2-HC-mediated increase in the activity of haem oxygenase, suggesting that the induction of haem oxygenase activity by this chalcone occurs at the transcriptional level. Investigation into the involvement of MAPK in the mechanism involved in the 2-HC-mediated induction of HO-1, indicated that the three MAPK pathways are not necessary for activation of haem oxygenase by 2-HC, our data is in agreement with data provided in the literature (Foresti et al., 2005). Furthermore, we established the

novel finding that the activation of the PI3K pathway is essential for the activation of haem oxygenase by 2-HC. It is worth mentioning that there was no positive control that demonstrates the effectiveness of MAPK inhibitors, and therefore this should be borne in mind when interpreting these results. Moreover, pre-treatment of cells with 2-HC protected BAEC from the oxidative damage-mediated by H2O2, this is in accordance of data in the literature (Foresti et al., 2005). Furthermore, 2-HC activated Nrf2, which resulted in the translocation of Nrf2 into the nucleus, this data is agreement with the previous data obtained by Al-caraz and co-workers (Alcaraz et al., 2004), furthermore, our group which showed that other plant-derived phytochemicals, induced HO-1 expression and resulted in the activation of Nrf2 (Balogun et al., 2003b),(Foresti et al., 2005). This further emphasizes the potent cytoprotective properties of this chalcone, having anti-oxidant properties per se as well as having inherent ability to strongly activate the endogenous cytoprotective pathways; this chalcone appears to possess antioxidant and free radical-scavenging characteristics. Thus, it is plausible that 2-HC could be used as a pharmacological preconditioning agent to modulate the expression of endogenous protective intracellular pathways in tissues exposed to oxidant-mediated injury.

In conclusion, our data show that chalcone is a potent inducer of HO-1 in BAEC, and it has beneficial effects in protection against oxidative stress. In addition, our data provided a mechanistic insight onto the effect of 2-HC on haem oxygenase. In view of these results, it is tempting to speculate that some beneficial effects of 2-HC might take place because of the intrinsic ability of this chalcone to up-regulate HO-1 and possibly other intracellular protective pathways.

# 4 HO-1 MEDIATES THE ANTI-INFLAMMATORY ACTIONS OF 2-HC IN RAW 264.7 MURINE MACROPHAGES

## 4.1 Introduction

The use of naturally-occurring compounds that possess intrinsic anti-inflammatory, and antioxidant properties and which can trigger the intracellular protective cascades offers a promising stratagem for therapeutic applications (Woodman and Chan, 2004). An interesting class of agents that might fill these criteria are the phytochemicals chalcones. 2-HC derivatives showed potent anti-inflammatory activity in various models of inflammation, including LPS-induced inflammatory response in macrophages (Ban et al., 2004) (Batt et al., 1993). Inflammation plays a role in cardiovascular pathological conditions (Schiffrin, 2002), upon stimulation by LPS, macrophages produce NO and pro-inflammatory cytokines such as IL-1 and TNF-α (Zembowicz and Vane, 1992). NO is generated by NO synthases and induces tissue injury at the inflammatory site (MacMicking et al., 1997). 2-HC derivatives have been shown to suppress the LPS-induced production of NO and TNF-α in the macrophage cell line RAW 264.7(Ban et al., 2004). It is postulated that chalcones exert their anti-inflammatory action by inhibiting the production of various inflammatory mediators such as cytokines and iNOS (Herencia et al., 2002), either by a direct inhibitory action on these mediators or inhibition of the transcription factors (NF-KB, AP-1) that regulate the production of the various mediators that are involved in the inflammatory response (Madan et al., 2000) (Ban et al., 2004). Furthermore, chalcones exert their cytoprotective actions via activation of a variety of transcriptional factors and by up-regulation of endogenous

cytoprotective pathways, such as phase-II enzymes, among them is the HO-1 (Alcaraz et al., 2004), as it was shown in different cell lines, in RAW 264.7 macrophages (Alcaraz et al., 2004) and in endothelial cells (Foresti et al., 2005). The HO-1 system plays a crucial role in the control of the inflammatory process (Alcaraz et al., 2003), this role is supported by several models of inflammation, in which HO-1 up regulation results in a significant reduction in inflammatory response (Zampetaki, 2003) (Berberat, 2005) (Wang, 2004) including oedema, leukocyte adhesion and migration, and production of cytokines (Alcaraz et al., 2003). HO-1 down regulates the pro-inflammatory cytokines (Otterbein et al., 2000) and the adhesion molecules (Wagener, 2001), furthermore, HO-1 up-regulates the anti-inflammatory cytokine IL-10 (Lee and Chau, 2002). On the other hand, pro-inflammatory cytokines and LPS have been shown to activate HO-1, in an adaptive response to inflammatory stress (Camhi et al., 1998), HO-1 end products biliverdin/bilirubin and CO have also been implicated in the modulation of a range of signalling pathways, including MAPK pathways by which they may modulate inflammatory processes (Morse et al., 2003) (Otterbein et al., 2000).

In the present study, the anti-inflammatory action of 2-HC was investigated in an LPS-induced model of inflammation in RAW 264.7 macrophages, and the role of HO-1 in this process was investigated.

## 4.2 Objectives

In this study, the potential anti-inflammatory action of 2-HC was investigated in an LPS-induced model of inflammation in RAW 264.7 macrophages; furthermore, the role of HO-1 in this process was elucidated. In addition, the signalling mechanisms involved in this process were investigated

## 4.3 Material and Methods

### 4.3.1 Reagents

All reagents were purchased from Sigma, unless otherwise specified. LPS is E-coli: serotype 026:B6 (Sigma). Tin Protoporphyrin IX (SnPPIX) was obtained from Porphyrin Products INC (Logan, Utah, USA). HO-1 and PI3K small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology.

### 4.3.2 Cell Culture

RAW 264.7 murine macrophages were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK) and cultured in DMEM containing, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and supplemented with 10% FBS. Cells were grown in 75-$cm^2$ flasks and maintained at 37°C in a humidified atmosphere of air and 5% $CO_2$.

### 4.3.3 Experimental Protocol

RAW 264.7 macrophages were treated with 2-HC (5, 15 and 30 μM) for 6 h, haem oxygenase activity and HO-1 protein expression were then determined. Haem oxygenase activity was also measured in cells exposed to 2-HC for 6 h, and the medium was then removed and replaced by fresh medium, cells were then incuabted for further 12 h. Furthermore, haem oxygenase activity was determined in cells treated for 24 h with LPS in the presence or absence of 2-HC. Cell viability was then assessed using LDH release and Alamar Blue Assay after 24 h-incubation with 2-HC alone, or following incubation of cells with the combination of 2-HC and LPS. In order to examine the potential anti-inflammatory action of 2-HC, macrophages were exposed for 24 h to LPS

(1 μg/ml) in the presence or absence of 2-HC (5, 15 and 30 μM), nitrite levels and iNOS protein expression were then determined at the end of the incubation. SiRNA for HO-1 were used to determine the involvement of HO-1. In a similar set of experiments, cells were treated with 2-HC for 6 h prior to incubation with LPS and nitrite production was measured after 24 h. The levels of TNF-α were also determined in cells exposed for 12 h to LPS (0.1 μg/ml) in the presence or absence of 2-HC. Similarly, TNF-α was measured in experiments conducted with SnPPIX (10 μM) and HO-1 siRNA. The participation of the MAPK pathway in the increase of haem oxygenase activity and HO-1 expression by 2-HC was assessed using PD098059 (ERK inhibitor, 25 μM), SB203580 (p38 inhibitor, 5 μM) and SP600125 (JNK inhibitor, 10 μM). An inhibitor of the PI3K pathway (LY294002, 25 μM) was also tested, in addition to PI3K siRNA. Furthermore, to investigate the effect of 2-HC on the translocation of the transcription factor NF-KB to the nucleus, cells were pre-incubated with 2-HC for 30 min, followed by treatment with LPS 1 μg/ml for 30 and 60 min. At the end of the experimental protocol, the nuclear extraction was performed.

### 4.3.4 Cell Viability/Alamar Blue Assay

Cell viability was determined using an Alamar Blue assay kit, it was carried out as previously described in Section 2.3.1.

### 4.3.5 LDH Assay

LDH release was measured using cytotoxicity detection kit (Roche) as previously described in section 2.3.2.

### 4.3.6 Haem Oxygenase Activity Assay

Haem oxygenase activity was determined at the end of each treatment as described previously in Section 2.4.3.

### 4.3.7 Western Blot Analysis for HO-1, iNOS and NF-KB

Cells were also analyzed for the determination of the protein expression for HO-1, iNOS and NF-KB by Western immunoblot technique as previously described in Section 2.5.5.

### 4.3.8 Determination of Nitrite

Nitrite levels were determined using the Griess method as previously described by our group (Chan and Kan, 1999). The measurement of this parameter is widely accepted as indicative of NO production. Briefly, the medium from treated cells cultured in 24-well plates was removed and placed into a 96-well plate (50 µl per well). The Griess reagent was added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0−300 µM in cell culture medium).

### 4.3.9 Measurement of TNF-α Production

TNF-α present in each sample was determined using a commercially available kit from R&D Systems (Abingdon, U.K.). The assay was performed according to the manufacturers' instructions, as prevoiusly described in Section 2.5.1.

### 4.3.10 Transfection of RAW 264.7 Macrophages with Small Interfering RNA for HO-1 and PI3K

RAW 264.7 macrophages were grown in twelve-well plates and transiently transfected with HO-1 or PI3K siRNA mixed with the appropriate transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions, as previously described in Section 2.5.4. After incubation at 37 °C for 30 h, cells were exposed to different reagents according to the Experimental Protocol.

## 4.4 Statistical Analysis

Differences among the groups were analyzed using one way ANOVA. Values were expressed as mean ± S.E.M., and differences between groups were considered to be significant at $p < 0.05$.

## 4.5 Results

### 4.5.1 2-HC Induces Haem Oxygenase Activity and HO-1 Expression in RAW 264.7 Macrophages

Firstly, we wanted to verify that 2-HC at the concentrations used in the present study are not toxic to the cells, for this purpose, LDH release was measured as an index of cell damage. Figure 4.4 A showed that incubation of macrophages for 24 h with increasing concentrations of 2-HC (5-30 μM) did not cause cytotoxicity. We have previously reported that 2-HC induces haem oxygenase activity and HO-1 expression in BAEC (Foresti et al., 2005). We wanted to establish the effect of 2-HC on HO-1 expression in RAW264.7 macrophages. As shown in (Figure 4.1), exposure of macrophages to 2-HC for 6 h resulted in a concentration-dependent increase in haem oxygenase activity (Figure 4.1 A) and HO-1 protein expression (Figure 4.1 B). We also assessed whether the effect on haem oxygenase activity was a long-lasting effect, for this purpose, haem oxygenase activity was measured in macrophages incubated with 2-HC for 6 h followed by 12 h to medium alone (Figure 4.1 C). Interestingly, haem oxygenase activity remained elevated even after removal of the chalcone, suggesting that macrophages need a long time to metabolize the compound. This possibility was further sustained by the fact that the extent of haem oxygenase activity was similar between cells incubated for 6 h with 2-HC and cells exposed to 6 h to 2-HC followed by 12 h incubation in medium (Figure 4.1 C).

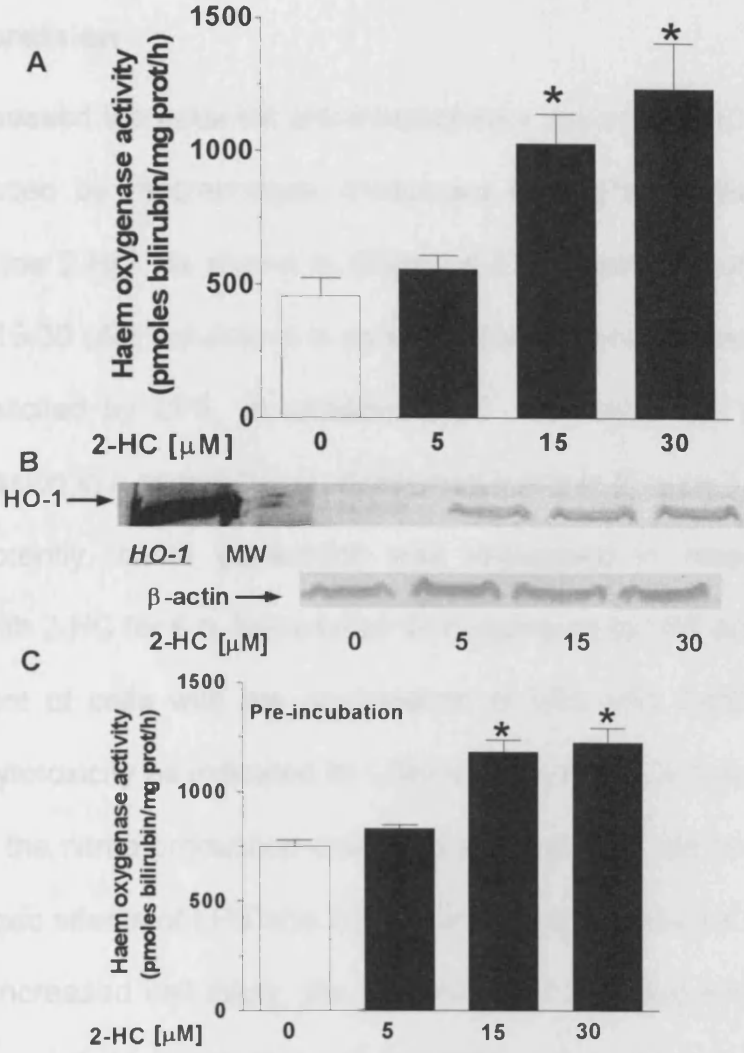



**Figure 4.1: The effect of 2-HC on haem oxygenase activity and HO-1 expression in RAW264.7 macrophages**

RAW 264.7 macrophages were exposed to various concentrations (5-30 μM) of 2-HC for 6 h. Haem oxygenase activity (A) and HO-1 protein expression (B) were determined at the end of the incubation. Cells treated with medium alone represented control (0). Western Blot is a representative of three independent experiments. Cells were exposed to various concentrations (5-30 μM) of 2-HC for 6 h followed by 12 h incubation in fresh medium alone (C). Haem oxygenase activity was measured at the end of the incubation (6+12 h). Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. control. β-actin was used as an internal control for equal loading *HO-1:* positive control recombinant HO-1 protein, MW: molecular weight marker.

### 4.5.2 2-HC Reduces LPS-Stimulated Nitrite Production and iNOS Expression

We next assessed the potential anti-inflammatory action of 2-HC by measuring nitrite produced by macrophages challenged with LPS, in the presence or absence of the 2-HC. As shown in (Figure 4.2 B), treatment of macrophages with 2-HC (15-30 μM) resulted in a concentration-dependent decrease in nitrite production-elicited by LPS. In addition, 2-HC decreased the LPS-stimulated iNOS expression in a concentration-dependent manner (Figure 4.2 B). Similarly, but less potently, nitrite generation was decreased in macrophages pre-incubated with 2-HC for 6 h, followed by 24 h exposure to LPS alone (Figure 4.3 B). Treatment of cells with the combination of LPS and 2-HC resulted in a significant cytotoxicity as indicated by LDH release (Figure 4.4 A); therefore, the reduction in the nitrite production and iNOS expression could be explained due to the cytotoxic effects of LPS and 2-HC. Furthermore, exposure of cells to LPS resulted in increased cell injury, the presence of 2-HC markedly reversed this effect (Figure 4.4 A). Intriguingly, cell viability assessed using Alamar Blue indicated that 2-HC at 30 μM, but not 5 or 15 μM, decreased the cellular metabolic activity (Figure 4.4 B). Co-incubation of cells with LPS and 2-HC at 30 μM further decreased cell viability by more than 50 %. Exposure of cells to LPS results in up-regulation HO-1 (Sawle et al., 2005), we determined the activity of haem oxygenase in macrophages exposed to LPS or to a combination of LPS and 2-HC for 24 h. Our results showed that cells incubated with LPS alone exhibited a significant increase in haem oxygenase activity (Figure 4.3 A), this effect was significantly increased in the presence of 2-HC in a concentration-dependent manner.

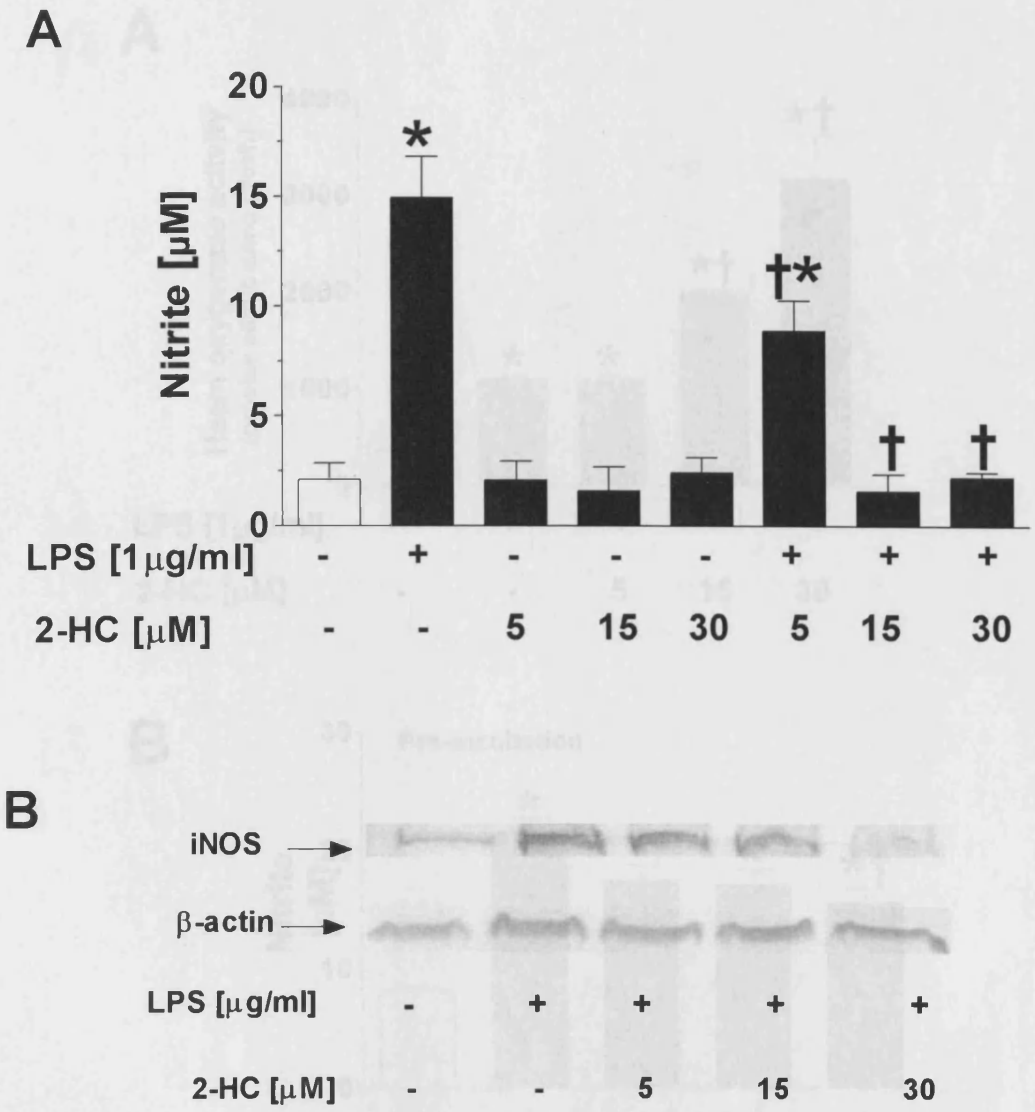



**Figure 4.2: 2-HC modulates LPS-stimulated nitrite production and iNOS expression**

RAW264.7 macrophages were exposed to LPS (1 μg/ml) in the presence or absence of 2-HC and nitrite production was determined after 24 h (A). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS. RAW264.7 macrophages were exposed to LPS (1 μg/ml) in the presence or absence of 2-HC and iNOS expression was determined at 24 h (B). Western Blots are representatives of three independent experiments. β-actin was used as an internal control for equal loading.

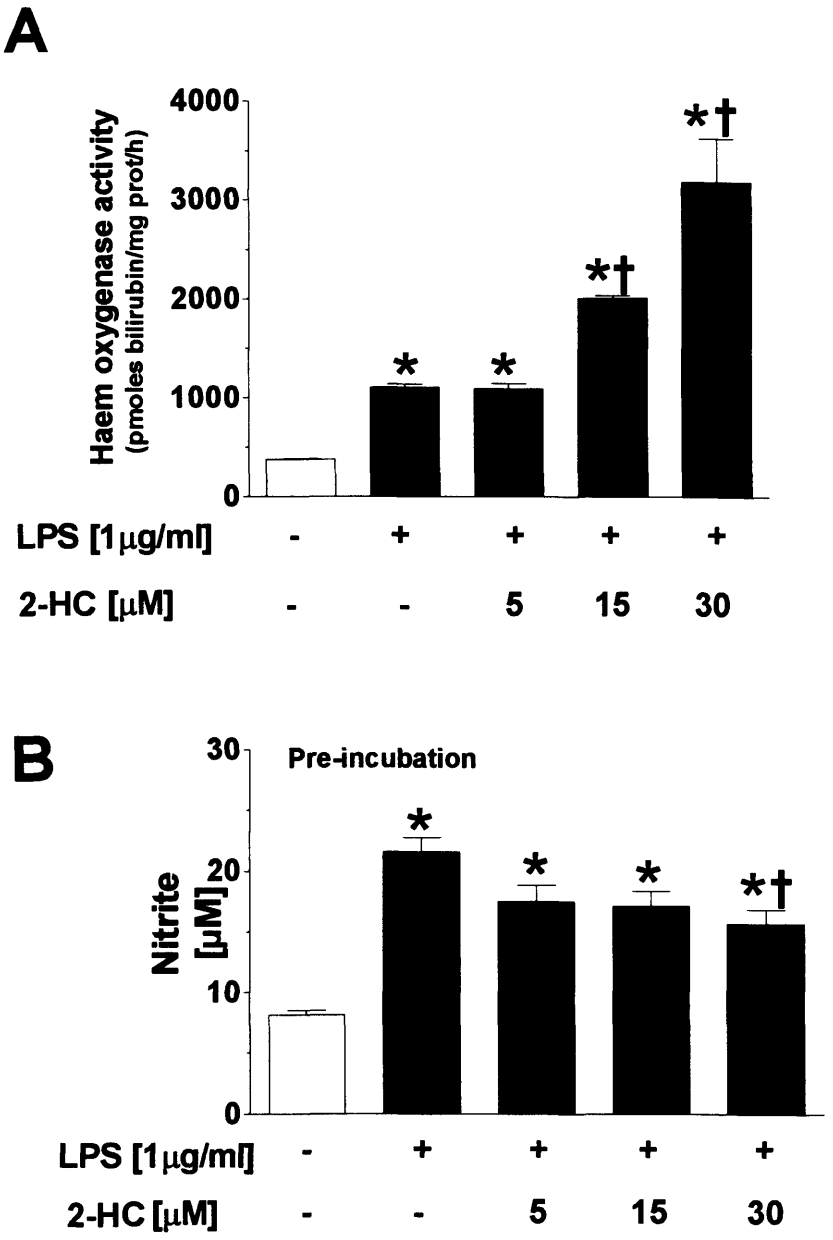



**Figure 4.3: Effect of pre-incubation with 2-HC on the LPS-mediated nitrite production**

RAW 264.7 macrophages were exposed to various concentrations (5-30 μM) of 2-HC in the presence of LPS 1 μg/ml for 24 h and haem oxygenase activity was measured at the end of the incubation (A). RAW 264.7 macrophages were exposed to 2-HC (5-30 μM) for 6 h followed by 24 h incubation in medium containing of LPS (1 μg/ml). Nitrite production was measured at the end of the incubation (6+24h) (B). Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS.

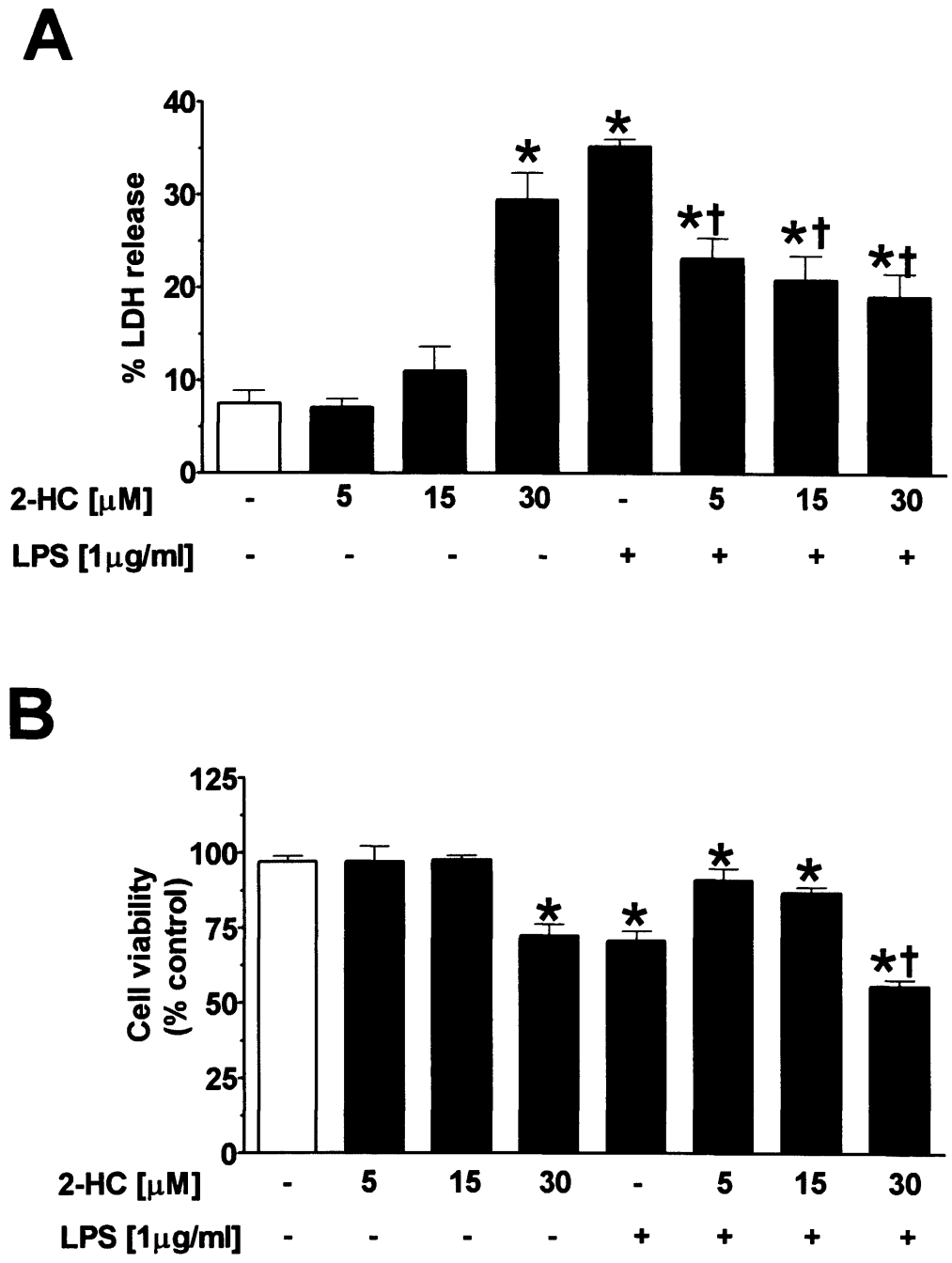



**Figure 4.4: Viability of cells exposed to 2-HC and LPS**

RAW 264.7 macrophages were exposed to LPS (1 μg/ml) for 24 h in the presence of 2-HC (5-30 μM) and cytotoxicity was determined using LDH release (A) and Alamar blue (B). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS.

### 4.5.3 2-HC Attenuates LPS-Stimulated TNF-α Production

Since TNF-α is a major cytokine involved in the inflammatory response triggered by LPS (Tracey, 1994), we tested the effect of 2-HC on LPS-induced TNF-α production. Treatment of macrophages with LPS resulted in a significant increase in TNF-α, 2-HC significantly decreased LPS-mediated TNF-α production in a concentration-dependent manner (Figure 4-5 A), inhibition of the haem oxygenase pathway with SnPPIX significantly prevented this effect (Figure 4-5 B).

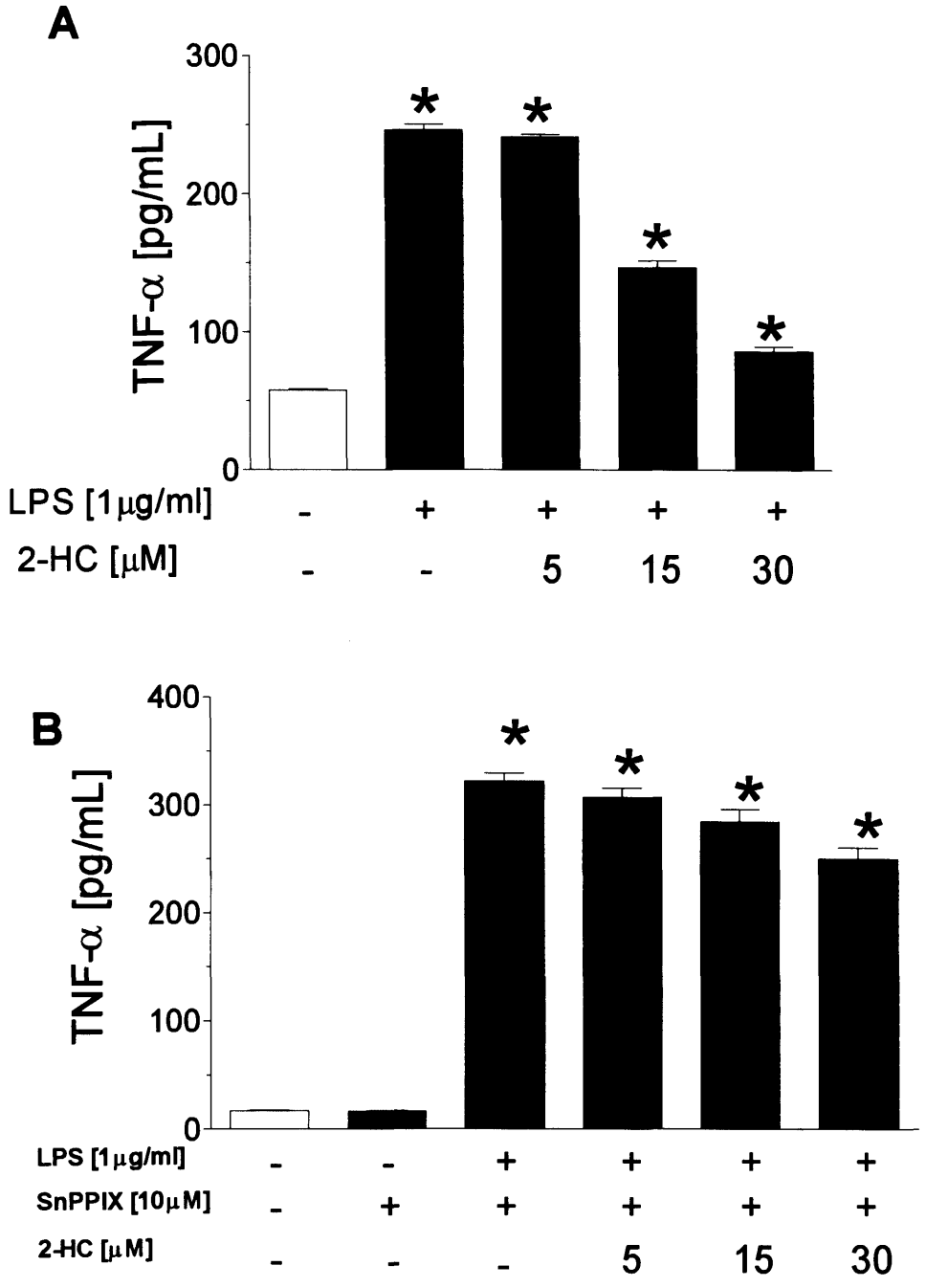



**Figure 4.5 : 2-HC modulates LPS-stimulated TNF-α production.**

RAW 264.7 macrophages were exposed to LPS (0.1 μg/ml) for 12 h in the presence or absence of 2-HC (5-30 μM) and TNF-α production was determined (A). Cells were exposed to LPS for 12 h in the presence or absence of 2-HC and SnPPIX (10 μM) and TNF-α production was determined (B). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. Control.

### 4.5.4 HO-1 Mediates the 2-HC Elicited Attenuation of Nitrite, iNOS Expression and TNF-α

Having established the anti-inflammatory effects of 2-HC, the involvement of the haem oxygenase pathway in the 2-HC-mediated reduction of nitrite production and TNF-alpha was then tested using siRNA for HO-1. In cells transfected with HO-1 siRNA, the effect of 2-HC on LPS-mediated increase in nitrite production (Figure 4.6 A), iNOS expression (Figure 4.8 B) and TNF-α (Figure 4.6 B) was abolished. This indicates that the activation of the HO-1 pathway is essential to 2-HC-mediated anti-inflammatory effects. It is interesting to note that in macrophages treated with HO-1 siRNA, the levels of nitrite and TNF-α production following treatment with LPS was 4-5 times higher than in cells with normal HO-1 expression, compare (Figure 4.6 A) with (Figure 4.2 A), and (Figure 4.6 B) with (Figure 4.5 A).

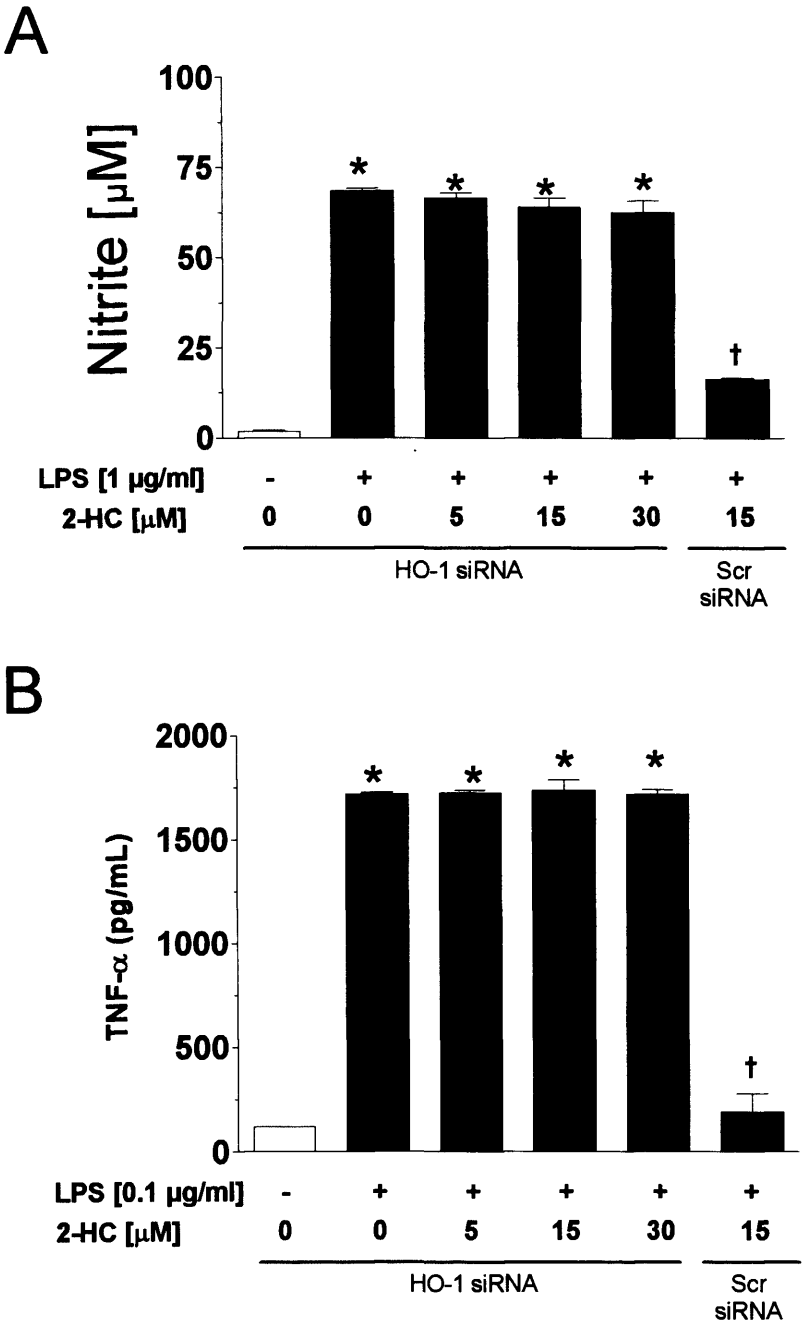



**Figure 4.6: HO-1 expression is required for the anti-inflammatory actions of 2-HC.** RAW 264.7 macrophages transfected with HO-1 siRNA were exposed to LPS (1 μg/ml) in the presence or absence of 2-HC and nitrite production was determined after 24 h (A). Cells transfected with HO-1 siRNA were exposed to LPS (0.1 μg/ml) for 12 h in the presence or absence of 2-HC and TNF-α production was determined (B). Cells treated with medium alone represent the control group (0 μM). Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. 0 μM (Control). Scr = scrambled siRNA.

### 4.5.5 The PI3K Pathway is Required for the Induction of Haem Oxygenase Activity and HO-1 Expression Mediated by 2-HC

To investigate the signalling cascade mediating the increase in haem oxygenase activity and HO-1 expression by 2-HC, we employed pharmacological antagonists of the MAPK and PI3K pathways. As observed previously with endothelial cells (Itoh et al., 1999), inhibition of ERK, JNK (Figure 4.7 A) or p38 (Figure 4.7 B) did not result in any significant effect on the 2-HC-mediated induction of haem oxygenase activity and HO-1 expression. However, blockade of the PI3K pathway completely prevented the 2-HC mediated up-regulation of HO-1 (Figure 4.7 B). To further support the role of PI3K-mediated induction of HO-1 by 2-HC, we used siRNA for the PI3K pathway; as shown in (Figure 4.8 A), 2-HC failed to induce HO-1 expression in macrophages treated with either HO-1 or PI3K siRNA.

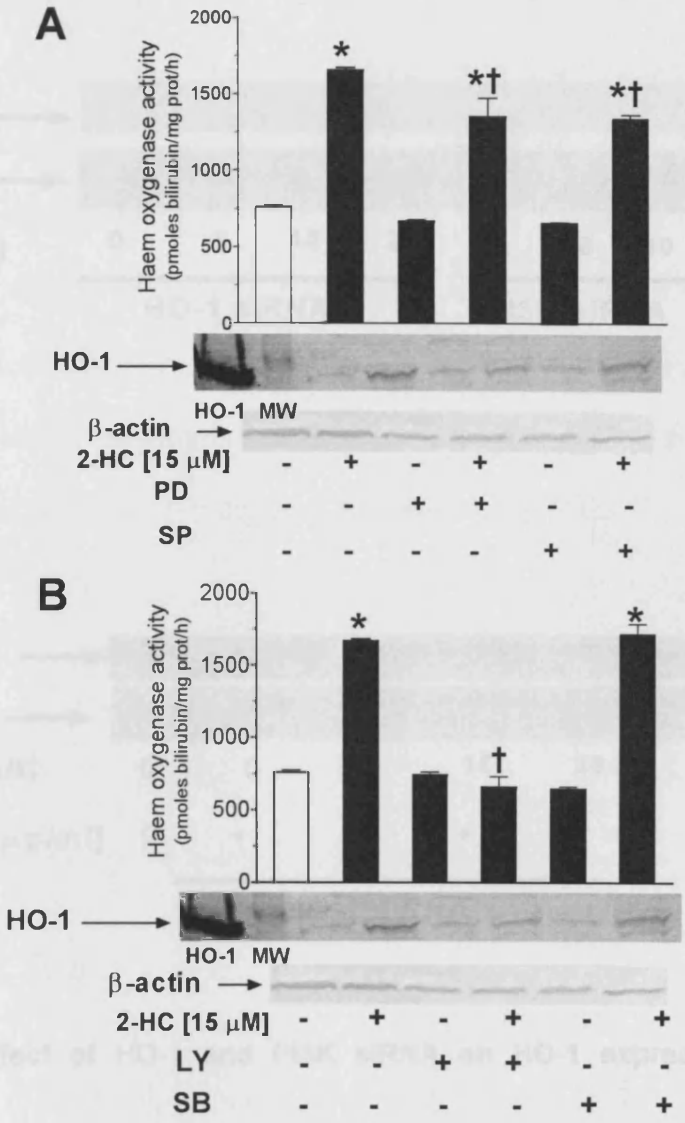



**Figure 4.7: Effect of MAPK inhibitors on the on the increase of haem oxygenase activity and HO-1 expression mediated by 2-HC**

RAW 264.7 macrophages were pre-treated for 30 min with PD 098,059 (ERK inhibitor, 25 μM) or SP 600125 (JNK inhibitor, 10 μM) in serum free-medium prior to exposure to 15 μM 2-HC for 6 h. Haem oxygenase activity and HO-1 expression were determined at the end of the incubation (A). Cells were pre-treated for 30 min LY 294002 (PI3K pathway inhibitor, 25 μM) or SB 203580 (p38 inhibitor, 5 μM) in serum free-medium prior to exposure to 15 μM 2-HC for 6 h (B). Haem oxygenase activity and HO-1 expression were determined at the end of the incubation. Bars represent the mean ± S.E.M. of 5-6 independent experiments, *P < 0.001 vs. Control, † P <0.001 vs. 2-HC alone. Western blot is representative of three independent experiments. β-actin was used as an internal control for equal loading. *HO-1:* positive control recombinant HO-1 protein, MW: molecular weight marker.

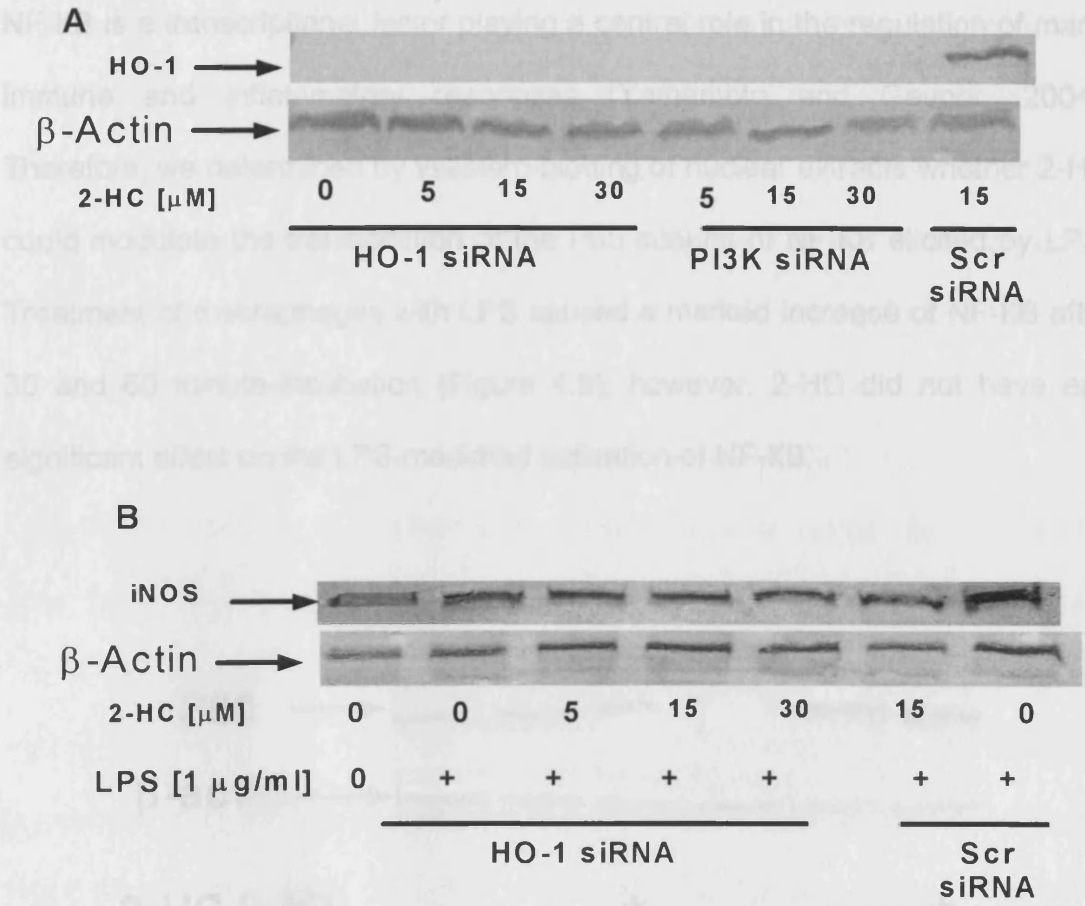



**Figure 4.8: Effect of HO-1 and PI3K siRNA on HO-1 expression and iNOS expression**

RAW 264.7 macrophages were transfected with HO-1 or PI3K siRNA and exposed to various concentrations (5-30 μM) of 2-HC for 6 h and HO-1 protein expression was determined (A). Cells transfected with HO-1 siRNA were also exposed to LPS in the presence or absence of 2-HC for 24 h and iNOS expression determined (B). Cells treated with medium alone represent the control group (0 μM). Western blots are representative of three independent experiments. Scr = scrambled siRNA.

### 4.5.6 Effect of 2-HC on the Nuclear Translocation of NF-KB

NF-KB is a transcriptional factor playing a central role in the regulation of many immune and inflammatory responses (Yamamoto and Gaynor, 2004). Therefore, we determined by Western blotting of nuclear extracts whether 2-HC could modulate the translocation of the P65 subunit of NF-KB elicited by LPS. Treatment of macrophages with LPS caused a marked increase of NF-KB after 30 and 60 minute-incubation (Figure 4.9), however, 2-HC did not have any significant effect on the LPS-mediated activation of NF-KB.

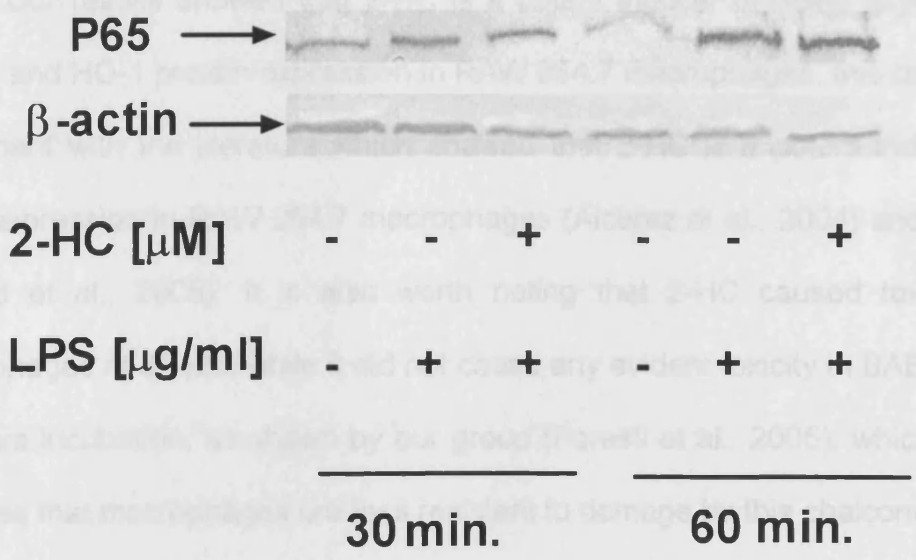



**Figure 4.9 Effect of 2-HC on the nuclear translocation of NF-KB elicited by LPS**
RAW 264.7 macrophages were pre-treated with 15 μM 2-HC for 30 min. followed by an additional 30 or 60 min. incubation with LPS (1 μg/ml). The translocation of the P65 subunit of NF-KB to the nucleus was determined in nuclear extracts by Western Blot. Western blots are representatives of three independent experiments. β-actin was used as an internal control for equal loading.

## 4.6 Discussion

In the present study, the anti-inflammatory properties of 2-HC were studied in an LPS-induced model of inflammation in RAW 264.7 macrophages. Recently, it was shown that many phytochemicals found in fruit and vegetables have the intrinsic ability to modulate endogenous cytoprotective pathways, including phase II response enzymes (Talalay and Fahey, 2001) among those is the HO-1 enzyme, which was found to be modulated by different phytochemicals, such as curcumin in renal epithelial cells (Balogun et al., 2003b) and in astrocytes (Scapagnini et al., 2002), Caffeic acid phenethyl ester in astrocytes (Scapagnini et al., 2002) and by carnosol in rat pheochromocytoma PC12 cells (Martin et al., 2004). Our results showed that 2-HC is a potent inducer of haem oxygenase activity and HO-1 protein expression in RAW 264.7 macrophages, this data is in agreement with the literature which showed that 2-HC is a potent inducer of HO-1 expression in RAW 264.7 macrophages (Alcaraz et al., 2004) and BAEC (Foresti et al., 2005). It is also worth noting that 2-HC caused toxicity in macrophages at 30 μM, while it did not cause any evident toxicity in BAEC after 24 hours incubation, as shown by our group (Foresti et al., 2005), which might indicates that macrophages are less resistant to damage by this chalcone. 2-HC derivatives were shown to suppress the LPS-induced production of nitrite and TNF-α in RAW 264.7 macrophage (Ban et al., 2004). Our results showed that 2-HC markedly the LPS-induced production of nitrite and iNOS expression in a concentration-dependent manner; these results are similar to the results obtained using 2-HC derivatives (Alcaraz et al., 2004) (Ban et al., 2004). However, cell viability tests showed that the co-incubation of LPS and 2-HC at 30 μM caused a marked toxicity in the cells after 24 h-incubation, so the

decrease in the nitrite production and iNOS protein expression at this particular concentration might be to toxicity in the cells. It is also interesting to note that pre-incubation with 2-HC prior to addition of LPS elicited a reduction in nitrite production, suggesting that 2-HC can exert delayed effects, even if is not present in the culture medium. Moreover, the effect of 2-HC on TNF-α, a crucial cytokine that is involved in the inflammatory response elicited by LPS (Anderson, 2004) (Tracey, 1994) was assessed. Our results showed that 2-HC inhibited the production of TNF-α in the cells challenged with LPS in a concentration-dependent manner, these results are similar to the results obtained using different derivatives of 2-HC (Alcaraz et al., 2004) (Ban et al., 2004), which indicates that this chalcone is a potent inhibitor of the key mediators of the pro-inflammatory cascade caused by LPS. We also demonstrated the novel finding that the anti-inflammatory effects of this chalcone are mediated by HO-1, since these effects were totally abolished in the presence of SnPPIX, a potent inhibitor of haem oxygenase activity, and HO-1 knockout cells using siRNA. To elucidate the transcriptional mechanisms involved in the inhibitory actions of 2-HC on LPS-induced production of nitrite and TNF-α, the effect of 2-HC on the activation of NF-KB, an essential transcription factor for the different inflammatory cytokines and iNOS expression (Yamamoto and Gaynor, 2004), was examined. Our results showed that 2-HC did not inhibit the LPS-induced translocation of the p65 subunit of NF-KB; these findings point out that the inhibition of nitrite production and iNOS expression by this compound is not mediated through the p65 subunit of NF-KB pathway. This is in disagreement with data in the literature, which showed that one of 2-HC derivatives inhibited the activation of NF-KB (Alcaraz et al., 2004). However, in

order to elucidate the effect of 2-HC on the activation of NF-KB further studies are needed, such as electrophoretic mobility shift assay (EMSA), and the assessment of the integrity of IKB (Ranjan et al., 2004).

To investigate the mechanisms of induction of HO-1 by 2-HC, we studied involvement of MAPK pathways (p38, ERK and JNK), in addition to the PI3K pathway. Data in the literature showed evidence of the involvement of MAPK pathways in the biological actions of the chalcones (Lee et al., 2002) (Frigo et al., 2002), for example, Butein (3,4,2',4'-tetrahydroxychalcone) inhibitory effects on NF-KB was mediated through the phosphorylation of ERK pathway (Lee, 2004). Our data suggest that the MAPKs pathway play a minor role in 2-HC–mediated induction of haem oxygenase activity and HO-1 expression. PI3K pathway is involved in a wide range of signaling pathways, mainly cell proliferation, cell death and apoptosis (Song et al., 2005), furthermore, it has been shown that PI3K is involved in the activation of phase II response enzymes (Lee et al., 2001), based on these findings and the diverse effects of PI3K, we examined whether this pathway is involved in the effect of 2-HC on haem oxygenase activity and HO-1 expression. In cells treated with LY294002 (a pharmacological inhibitor of the PI3K pathway) or PI3K siRNA, 2-HC-induced haem oxygenase activity and HO-1 protein expression were decreased significantly. Our data demonstrated, for the first time, the involvement of the PI3K pathway in the 2-HC-mediated induction of haem oxygenase activity and HO-1 protein expression. In conclusion, this study demonstrated that 2-HC is an anti-inflammatory compound that down regulates the inflammatory response in the LPS-macrophages model, in a mechanism which involves the haem oxygenase system.

# 5 CARBON MONOXIDE-RELEASING MOLECULES MODULATE LPS-INDUCED INFLAMMATORY RESPONSE IN RAW 264.7 MURINE MACROPHAGES

## 5.1 Introduction

CO has emerged in the last decade as a novel modulatory gas which exhibits a wide range of cellular functions including anti-inflammatory (Otterbein et al., 2000), and anti-apoptotic (Zhang et al., 2003) and has been shown to be beneficial in many models of inflammation (Morse, 2003) and I/R injury (Nakao et al., 2003) (Nakao et al., 2005). Furthermore, it has been demonstrated that CO exhibited inhibitory effects on the LPS-induced inflammatory response *in vivo* (Sarady et al., 2004), and *in vitro* (Morse et al., 2003). Recently, transitional metal carbonyls have been identified as potential CO-releasing molecules (CO-RMs) to facilitate the delivery of CO into biological systems (Motterlini, 2005). These compounds can release CO at controllable levels, which facilitate their use in therapeutic applications. In the present study, the effect of two novel water soluble CO-RMs, i.e. CORM-43 and CORM-319 on the inflammatory response in a model of inflammation in murine macrophages was examined. Previously, other CO-RMs have been studied in various experimental models and showed potent vaso-dilatory effects in isolated rat aortic rings (Foresti, 2004) and protective actions *in vitro* and *in vivo* models of cardiac I/R injury (Clark, 2003) as well as exhibiting anti-inflammatory effects (Sawle et al., 2005). LPS, a constituent of the gram negative bacterial cell wall, is a potent activator of macrophages, and induces a cascade of inflammatory responses which leads to the production of pro-inflammatory cytokines like TNF-α and IL-1

(Raetz et al., 1991). This is accompanied by increased expression and activity of iNOS, with a concomitant rise in NO generation production released during phagocytosis (Nathan, 1997). However, excessive production of NO can result in adverse effects, like tissue damage and hypotension, which could lead to endotoxic shock and death (Boscβ, 2005). NO levels in cells must therefore be tightly-regulated (Yamamoto and Gaynor, 2004). The inflammatory response to LPS is orchestrated by a group of transcriptional factors, including, NF-KB (Guha and Mackman, 2001). Activation of I-KB (which anchors the NF-KB in the cytoplasm in un-stimulated cells) by different stimuli such as ROS, cytokines and LPS leads to the phosphorylation and proteolysis of I-KB, which leads in turn to the translocation of NF-KB into the nucleus (Verma and Stevenson, 1997). NF-KB has been implicated in the pathogenesis of a number of inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease and atherosclerosis (Yamamoto and Gaynor, 2004). It has been shown that NF-KB plays a critical role in the regulation of iNOS (Surh et al., 2001). It was shown previously that CO blocked the LPS-induced NF-KB activation (Sarady et al., 2002).

In the present study, two new CO-RMs, CORM-43 and CORM-319 were investigated *in vitro* model of inflammation using LPS-induced inflammation in RAW 264.7 murine macrophages, and the different mechanisms involved in this process were investigated.

## 5.2 Objectives

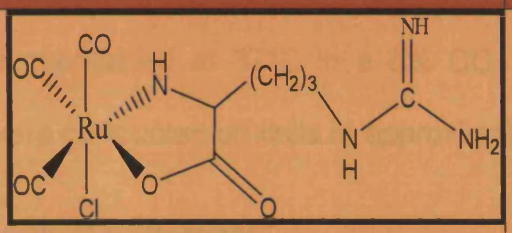
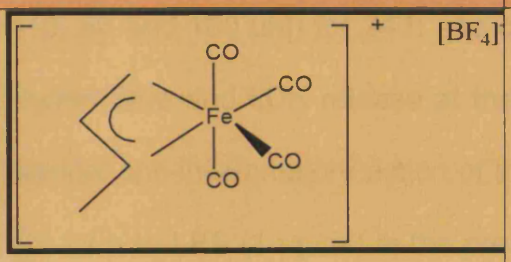
The purpose of this study is to investigate the potential anti-inflammatory effects of CORM-43 and CORM-319 *in vitro* using the LPS-induced responses of inflammation in RAW 264.7 murine macrophages.

## 5.3 Material and Methods

### 5.3.1 Preparation of Reagents

CORM-43 and CORM-319 (Table 5-1) were freshly prepared as a 10 mM stock solution in $dH_2O$. Inactive forms of each CO-RM (negative controls) were also used in some experiments and they were prepared as follows: CORM-43 and CORM-319 were inactivated by adding cell culture medium to the compound and leaving it for 24 h at 37°C in a 5% $CO_2$ humidified atmosphere to liberate CO. The iCORM-43 and iCORM-319 solutions were finally bubbled with nitrogen to remove the residual CO present in the solution. All other chemicals were reagent obtained from Sigma unless otherwise stated.

**Table 5-1: Characteristics of CORM-43 and CORM-319**

| Code | Structure/formula | Transition metal | Kinetics of CO release |
|---|---|---|---|
| CORM-43 |  **$Ru(CO)_3Cl$-arginine** | Ruthenium | Instant |
| CORM-319 |  **$[Fe(\eta\text{-}C_3H_5)(CO)_4][BF_4]$** | Fe | Steady, plateau At 40 minutes |

### 5.3.2 Cell Culture

Murine RAW264.7 macrophages were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, U.K.) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin and streptomycin. Cultures were maintained at 37°C in a 5% $CO_2$ humidified atmosphere and experiments were conducted on cells at approximately 80–90% confluence

### 5.3.3 Experimental Protocol

RAW 264.7 macrophages were exposed to various concentrations of CORM-43 or CORM-319 (10, 50 and 100 μM) for 24 h and cell viability was assessed by Alamar Blue, Trypan Blue and LDH release at the end of the experiments. To examine the potential anti-inflammatory action of these CO-RMs, macrophages were exposed for 24 h to LPS (1 μg/ml) in the presence or absence of CORM-43 or CORM-319 (10, 50 and 100 μM), nitrite levels and iNOS protein expression were then determined at the end of the incubation. Cytotoxicity was also determined in cells exposed to LPS (1 μg/ml) and CORM-43 or CORM-319 at 24 h. Experiments were repeated with the negative controls iCORM-43 and iCORM-319 to assess whether the effects observed were due to the CO liberated by the CO-RMs or caused by other components of the molecules. The effect of CO-RMs and their inactive forms on the haem oxygenase pathway was also investigated. Specifically, cells were treated for 6 h or 18 h in the presence of 10, 50 and 100 μM CO-RMs and haem oxygenase activity was then determined. The levels of TNF-α and IL-10 were also determined in cells exposed for 24 h to LPS (0.1 μg/ml) in the presence or absence of CO-RMs or their inactive forms. Furthermore, to investigate the effect of CO-RMs on the translocation of the transcription factor NF-KB to the nucleus, cells were pre-

incubated with CORM-43 or its inactive forms for 30 min, followed by treatment with LPS 1 μg/ml for 30 and 60 min. at the end of the experimental protocol.

### 5.3.4 Detection of CO Release

The release of CO from CORM-43 and CORM-319 or iCORM-43 and iCORM-319 was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) as previously described (Motterlini et al., 2002a). The release of CO from metal carbonyl complexes was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO). The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient=15.4 mmol/L-1 · cm-1). Myoglobin solutions (66 μmol/L final concentration) were prepared fresh by dissolving the protein in 0.04 mol/L phosphate buffer (pH 6.8). Sodium dithionite (0.1%) was added to convert myoglobin to deoxy-Mb prior to each reading. In contrast, CO released from CORM-43 was quantified by adding aliquots of stock solutions (10 μL) of the carbonyl complex in water directly to the myoglobin solution. All the spectra were measured using a Helios α-spectrophotometer.

### 5.3.5 Cell Viability/Alamar Blue Assay

Cell viability was determined using an Alamar Blue assay kit, it was carried out as previously described in Section 2.3.1.

### 5.3.6 LDH Assay

Extracellular, i.e., released, LDH activity was measured using cytotoxicity detection kit (Roche) as previously described in section 2.3.2.

### 5.3.7 Trypan Blue Assay

Trypan Blue exclusion was performed as previously described in section 2.3.3.

### 5.3.8 Haem Oxygenase Activity Assay

Haem oxygenase activity was determined at the end of each treatment as described previously in Section 2.4.3.

### 5.3.9 Western Blot Analysis for iNOS and NF-KB

Cells were also analyzed for the determination of the protein expression for iNOS and NF-KB by Western immunoblot technique as previously described in Section 2.5.5.

### 5.3.10 Determination of Nitrite

Nitrite levels were determined using the Griess method as previously described in Section 2.4.4.

### 5.3.11 Measurement of TNF-α Production

TNF-α present in each sample was determined using a commercially available kit from R&D Systems as previously described in Section 2.5.1.

### 5.3.12 Determination of IL-10 Levels

IL-10 present in each sample was determined using commercially available kit from R&D Systems in Section 2.5.2.

## 5.4 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with the Bonferroni test. Differences were considered to be significant at $P<0.05$.

## 5.5 Results

### 5.5.1 Detection of CO Release from CORM-43

The rate and amount of CO liberated from CORM-43 were measured in the cell culture medium (DMEM), which was used for cell incubation. In this study, we tested the ability of CORM-43 to release CO at 37 °C in DMEM and then we measured the kinetic of CO release from CORM-43 as described in the Materials and Methods (Section 2.4.5). As shown in Figure 5.1A, we found that the addition of the inactive form (iCORM-43) to deoxymyoglobin dissolved in DMEM at 37 °C did not produce any detectable MbCO over a 60 min period. However, the addition of CORM-43 (20, 40, and 60 μM) rapidly increased MbCO formation and reached a maximal level within minutes of release. For instance, after 60 min incubation with CORM-43, MbCO concentration was 14, 31, and 40 μM at 20, 40, and 60 μM CORM-43 (Figure 5.1A), which indicates that CORM-43 liberate approximately 0.75 mole of CO per 1 mole of compound. Absorption spectrum of MbCO after interaction of myoglobin with CO released from 20, 40, and 60 μM CORM-43, and 60 μM CORM-43 were compared to saturated MbCO (Figure 5.1B).

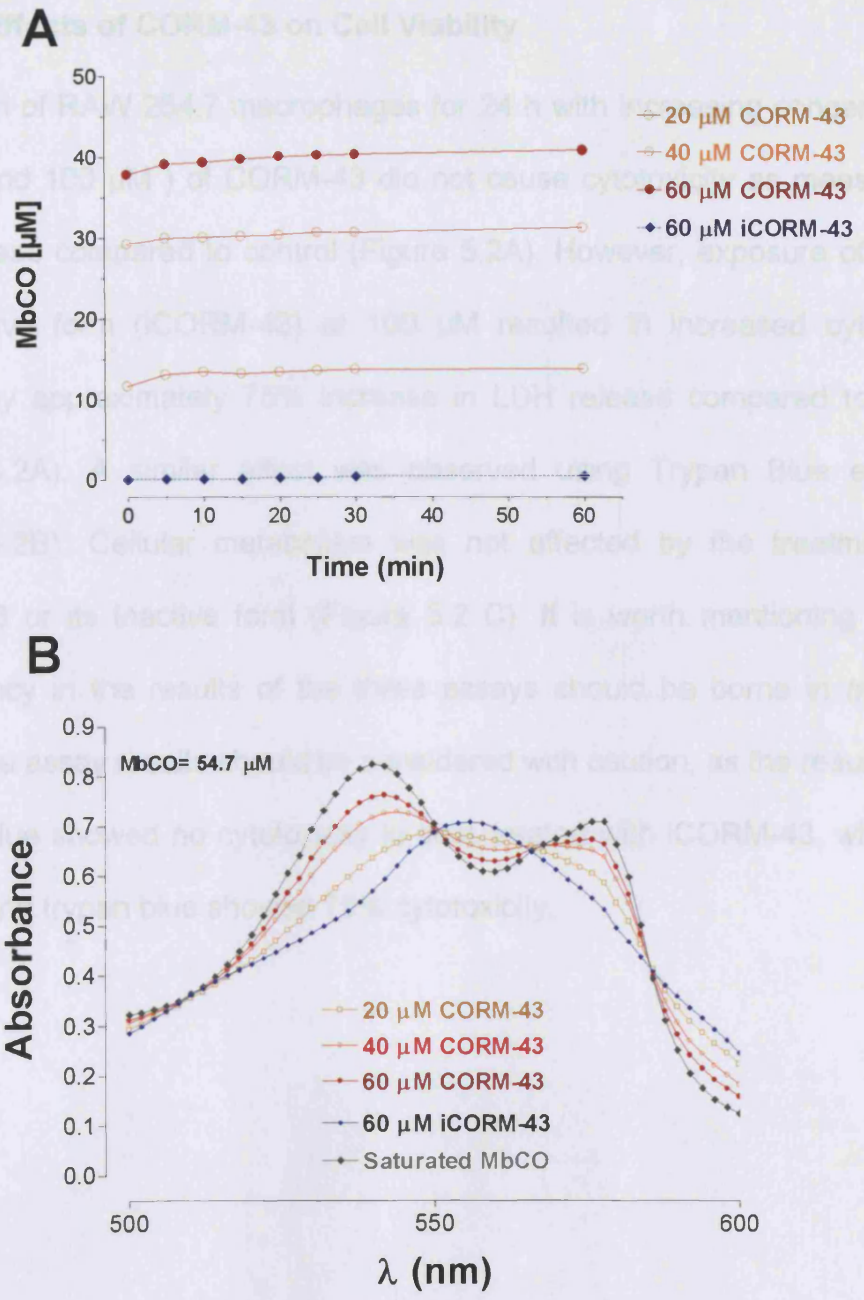



**Figure 5.1: Detection of CO released from CORM-43 in DMEM medium**

(A) Time course of CO released from 20, 40, and 60 µM CORM-43 after incubation in DMEM medium (pH=7.4) at 37°C. The amount of CO released was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin (MbCO). (B) Spectra of MbCO formation at 60 min after addition of CORM-43 to myoglobin solution. Each line represents the mean ± SEM of 2 independent experiments. The rate of CO release was calculated from the fitted curves. iCORM-43, which does not release CO, was used as negative control.

### 5.5.2 Effects of CORM-43 on Cell Viability

Incubation of RAW 264.7 macrophages for 24 h with increasing concentrations (10, 50 and 100 μM ) of CORM-43 did not cause cytotoxicity as measured by LDH release compared to control (Figure 5.2A). However, exposure of cells to the inactive form (iCORM-43) at 100 μM resulted in increased cytotoxicity evident by approximately 75% increase in LDH release compared to control (Figure 5.2A). A similar effect was observed using Trypan Blue exclusion (Figure 5.2B). Cellular metabolism was not affected by the treatment with CORM-43 or its inactive form (Figure 5.2 C). It is worth mentioning that the discrepancy in the results of the three assays should be borne in mind, the alarm blue assay results should be considered with caution, as the results of the Alamar blue showed no cytotoxicity in cells treated with iCORM-43, while LDH release and trypan blue showed 75% cytotoxicity.

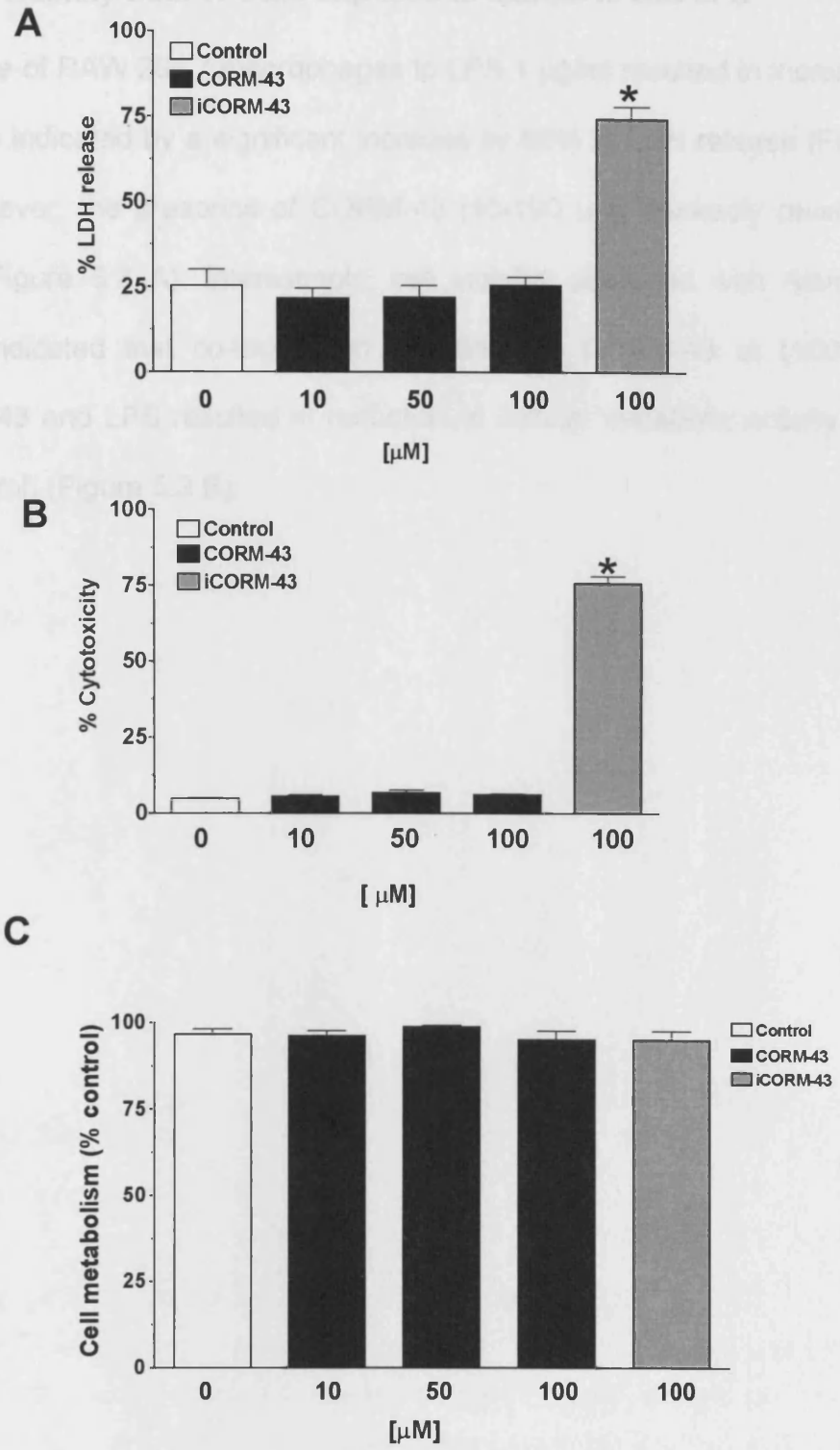



**Figure 5.2: Viability of cells exposed to CORM-43.**

RAW 264.7 macrophages were exposed for 24 h in the presence of CORM-43 (10-100 μM). Cell viability was then determined using a LDH release (A) Trypan Blue exclusion (B) and Alamar Blue (C). Bars represent the mean ± S.E.M. of 6 independent experiments, * $P < 0.001$ vs. control.

### 5.5.3 Viability Data of Cells Exposed to CORM-43 and LPS

Exposure of RAW 264.7 macrophages to LPS 1 μg/ml resulted in increased cell injury as indicated by a significant increase by 60% in LDH release (Figure 5.3 A). However, the presence of CORM-43 (10-100 μM) markedly reversed this effect (Figure 5.3 A). Interestingly, cell viability assessed with Alamar Blue assay indicated that co-incubation of cells with CORM-43 at (100 μM) or iCORM-43 and LPS resulted in reduction in cellular metabolic activity (80% of the control) (Figure 5.3 B).

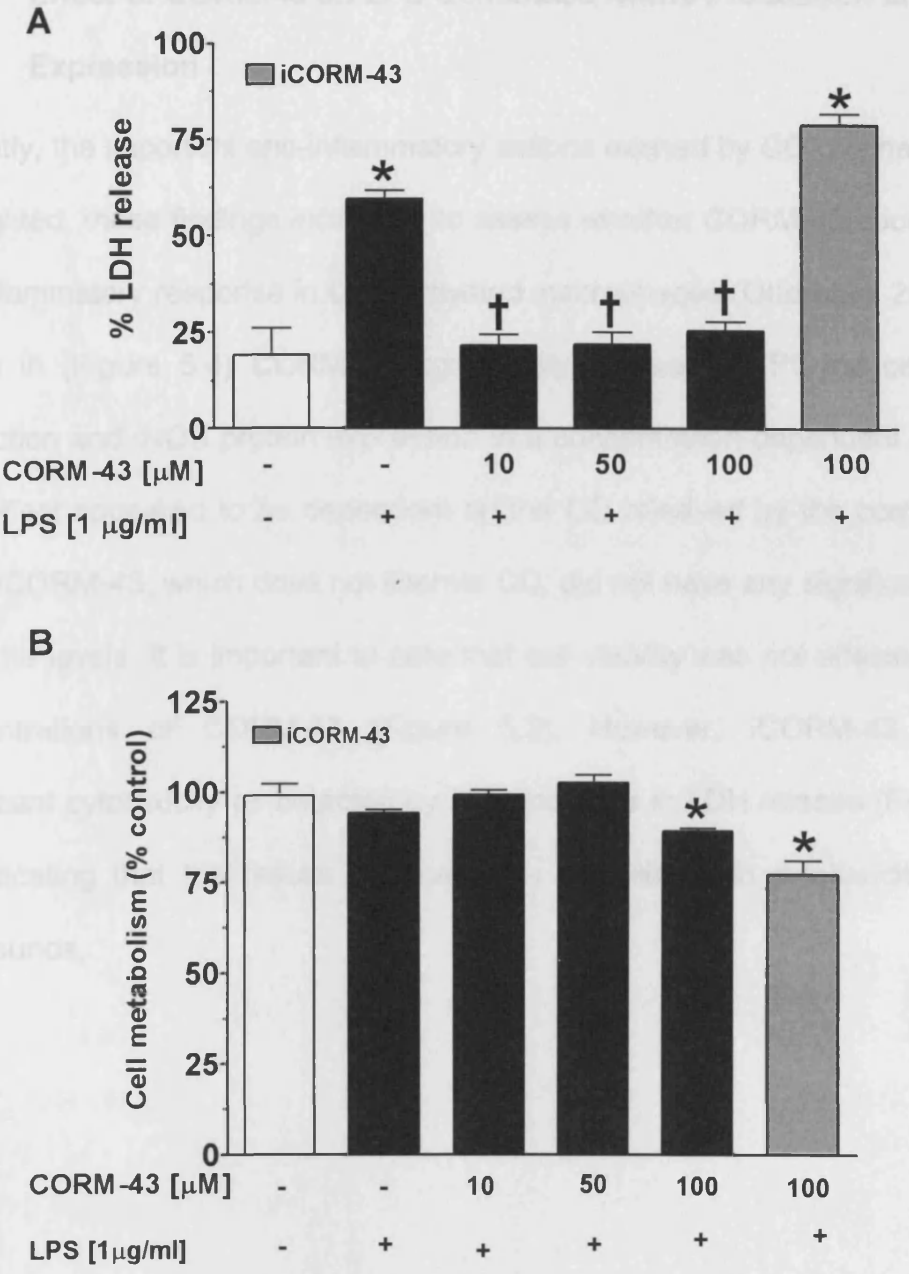



**Figure 5.3 : Viability of cells exposed to CORM-43 and LPS**

RAW 264.7 macrophages were treated with CORM-43 (10-100 μM) and LPS 1μg/ml for 24 h, cell viability was then determined using a LDH release (A) Trypan Blue exclusion (B) and Alamar Blue (C). Bars represent the mean ± S.E.M. of 6 independent experiments, * $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS.

### 5.5.4 Effect of CORM-43 on LPS-Stimulated Nitrite Production and iNOS Expression

Recently, the important anti-inflammatory actions exerted by CO gas have been highlighted, these findings incited us to assess whether CORM-43 could inhibit the inflammatory response in LPS-activated macrophages (Otterbein, 2002). As shown in (Figure 5.4) CORM-43 significantly attenuated LPS-induced nitrite production and iNOS protein expression in a concentration-dependent manner. This effect appeared to be dependent on the CO released by the compounds, since iCORM-43, which does not liberate CO, did not have any significant effect on nitrite levels. It is important to note that cell viability was not affected by the concentrations of CORM-43 (Figure 5.3). However, iCORM-43 caused significant cytotoxicity as detected by 75% increase in LDH release (Figure 5.3 A) indicating that the results observed are not related to cytotoxicity of the compounds.

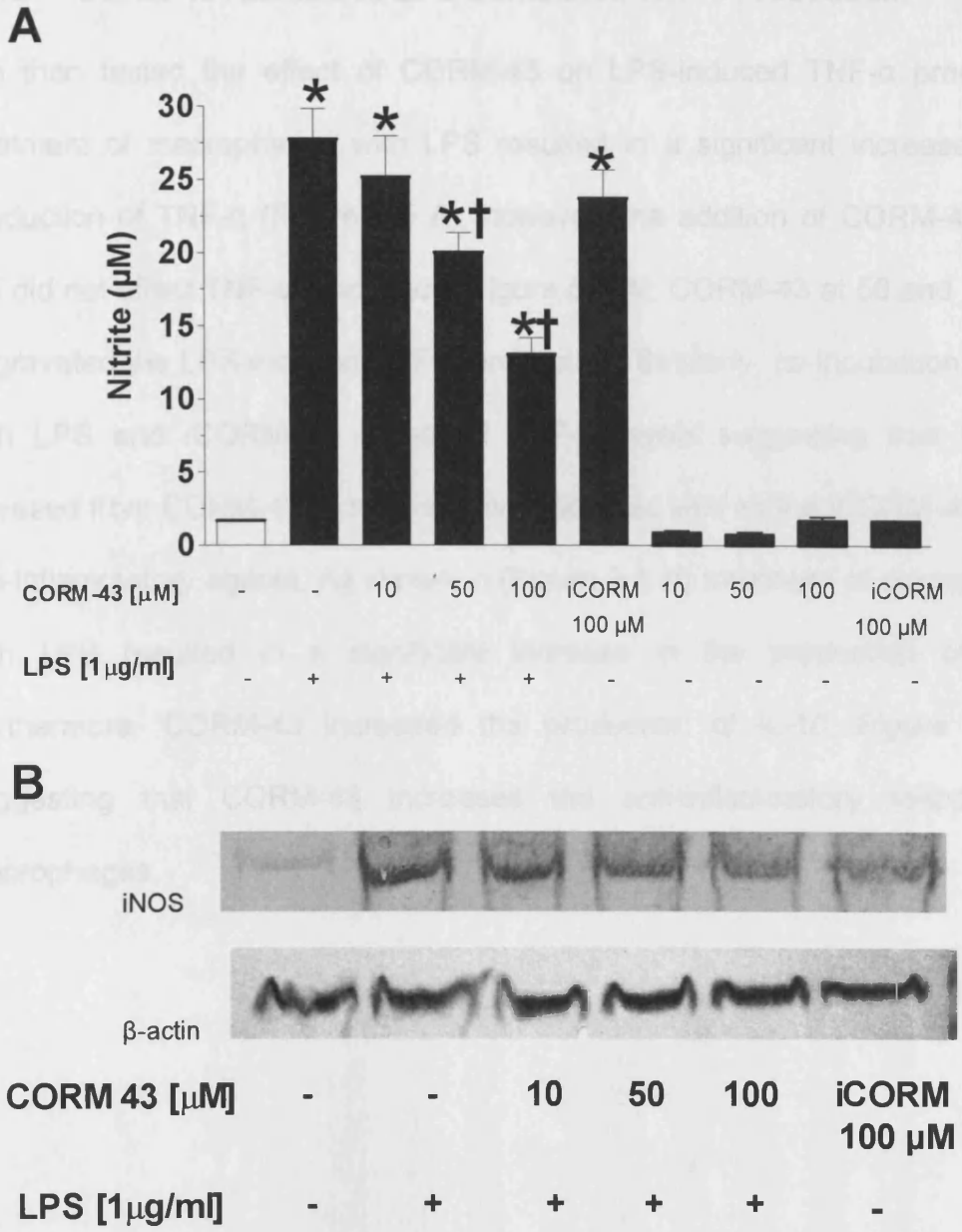



**Figure 5.4: CORM-43 modulates LPS-stimulated nitrite production and iNOS expression.**

RAW264.7 macrophages were exposed to various concentrations (10-100 μM) of CORM-43 or iCORM-43 (100 μM) in the presence of LPS 1 μg/ml for 24 h. Nitrite production (A) and iNOS expression (B) were then determined at 24 h. Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS. Western Blot is a representative of three independent experiments. β-actin was used as an internal control for equal loading.

### 5.5.5 CORM-43 Attenuates LPS-Stimulated TNF-α Production.

We then tested the effect of CORM-43 on LPS-induced TNF-α production, treatment of macrophages with LPS resulted in a significant increase in the production of TNF-α (Figure 5.5 A), however, the addition of CORM-43 at 10 μM did not affect TNF-α production (Figure 5.5 A). CORM-43 at 50 and 100 μM aggravated the LPS-induced TNF-α production. Similarly, co-incubation of cells with LPS and iCORM-43 increased TNF-α levels suggesting that the CO released from CORM-43 at high concentrations as well as the iCORM-43 act as pro-inflammatory agents. As shown in (Figure 5.5 B) treatment of macrophages with LPS resulted in a significant increase in the production of IL-10. Furthermore, CORM-43 increased the production of IL-10, Figure 5.5 B), suggesting that CORM-43 increases the anti-inflammatory response in macrophages.

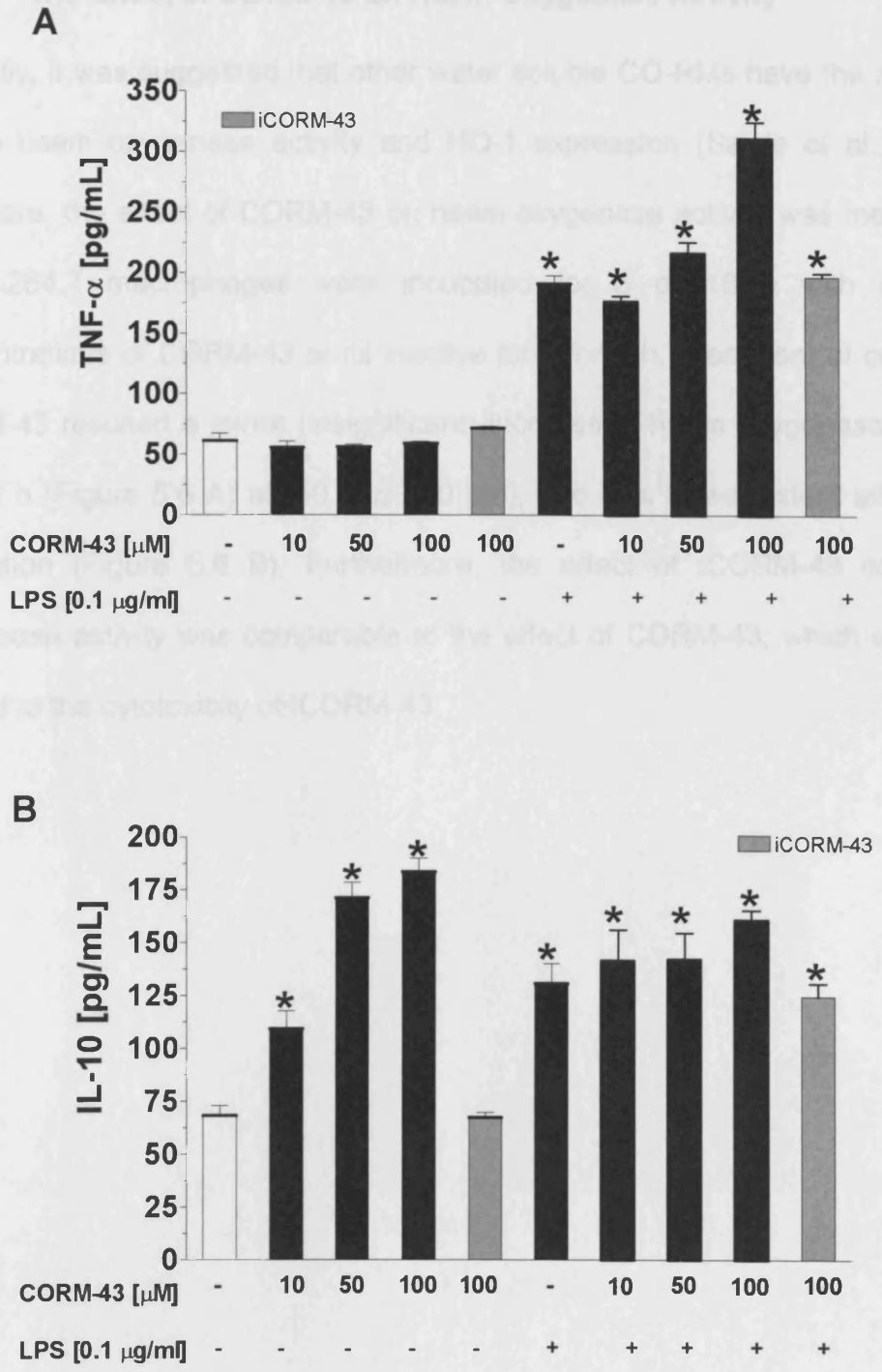



**Figure 5.5: CORM-43 attenuates LPS-stimulated TNF-α production and increases the production of IL-10.**

RAW 264.7 macrophages were exposed to LPS (0.1 μg/ml) for 24 h in the presence or absence of CORM-43 (10-100 μM), TNF-α (A) and IL-10 production (B) were determined. Bars represent the mean ± S.E.M. of 6 independent experiments, * P < 0.001 vs. Control.

### 5.5.6 The Effect of CORM-43 on Haem Oxygenase Activity

Recently, it was suggested that other water soluble CO-RMs have the ability to induce haem oxygenase activity and HO-1 expression (Sawle et al., 2005). Therefore, the effect of CORM-43 on haem oxygenase activity was measured. RAW 264.7 macrophages were incubated for 6 or 18 h with different concentrations of CORM-43 or its inactive form for 6 h. Treatment of cells with CORM-43 resulted a minor (insignificant) increase in haem oxygenase activity after 6 h (Figure 5.6 A) at (50 and 100 μM), and to a lesser extent after 18 h incubation (Figure 5.6 B). Furthermore, the effect of iCORM-43 on haem oxygenase activity was comparable to the effect of CORM-43, which could be related to the cytotoxicity of iCORM-43.

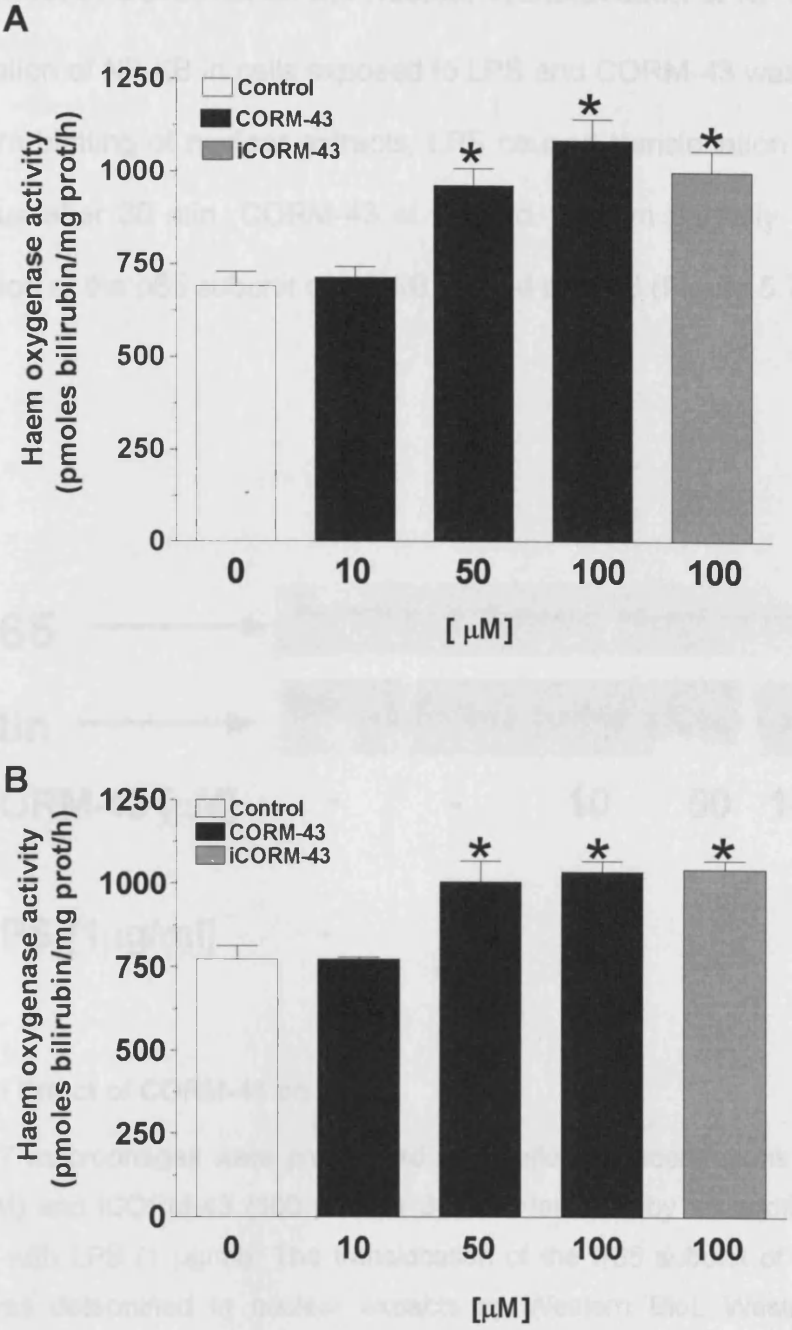



**Figure 5.6: The effect of CORM-43 on haem oxygenase activity in RAW264.7 macrophages.**

RAW 264.7 macrophages were exposed to various concentrations (10-100 μM) of CORM-43 for 6 h (A) or 18 h (B) and haem oxygenase activity was determined at the end of the incubation. Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.05$ vs. 0 μM.

### 5.5.7 Effect of CORM-43 on the Nuclear Translocation of NF-KB

The activation of NF-KB in cells exposed to LPS and CORM-43 was determined by Western blotting of nuclear extracts; LPS caused translocation of NF-KB to the nucleus after 30 min. CORM-43 at 50 and 100 μm partially inhibited the translocation of the p65 subunit of NF-KB elicited by LPS (Figure 5.7).

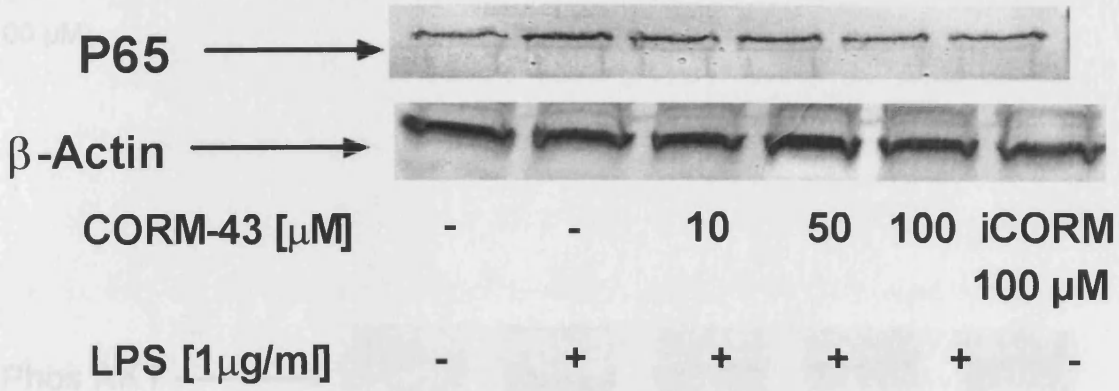



**Figure 5.7: Effect of CORM-43 on NF-KB**

RAW 264.7 macrophages were pre-treated with various concentrations of CORM-43 (10-100 μM) and iCORM-43 (100 μM) for 30 min. followed by an additional 30 min. incubation with LPS (1 μg/ml). The translocation of the P65 subunit of NF-KB to the nucleus was determined in nuclear extracts by Western Blot. Western blot is a representative of three independent experiments. β-actin was used as an internal control for equal loading.

### 5.5.8 The effect of CORM-43 on the phosphorylation of the AKT pathway

It was demonstared that CO targets the AKT pathway (Fujimoto et al., 2004), RAW 264.7 macrophages were pre-treated with various concentrations of CORM-43 (10-100 μM) and iCORM-43 (100 μM) for 30 min. The phosphorylation of AKT was then determined by Western Blot, Our results showed that treatment of RAW 167.4 macrophages with various concentrations (10-100) of CORM-43 resulted in the phosphorylation of the AKT pathway; however, this effect was not observed when cells were exposed to iCORM-43 (100 μM).

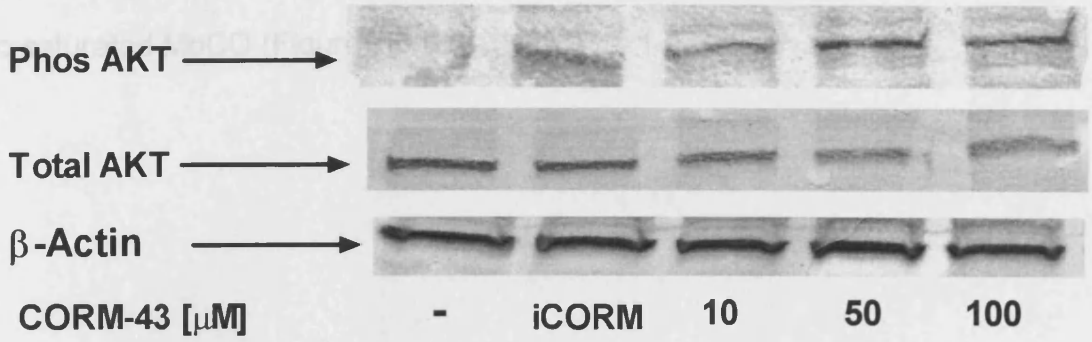



**Figure 5.8: Effect of CORM-43 on the phosphorylation of AKT**

RAW 264.7 macrophages were pre-treated with various concentrations of CORM-43 (10-100 μM) and iCORM-43 (100 μM) for 30 min. The phosphorylation of AKT was then determined by Western Blot. Western blot is a representative of three independent experiments. β-actin was used as an internal control for equal loading.

### 5.5.9 Detection of CO Release from CORM-319

Using the myoglobin assay to detect the formation of MbCO as described in Chapter 2 (Section 2.4.5), we tested the ability of 10, 20, 40, and 60 μM CORM-319 to release CO at 37 °C in the cell culture medium (DMEM). Here, we found that the rate of CO release by CORM-319 in DMEM at 37 °C directly correlates with the concentrations used (Figure 5.9). Specifically, the calculated rates of CO release were 0.14, 0.31, 0.43, and 0.60 nmol/min for 10, 20, 40 and 60 μM CORM-319, respectively. Predictably, the inactive compound (iCORM-319) did not release any detectable CO in the DMEM at 37 °C (Figure 5.9A). Interestingly, the increase in MbCO formation after addition of 60 μM CORM-319 to DMEM reached almost a maximal level after 40 min.

Absorption spectrum of MbCO after interaction of myoglobin with CO released from 10, 20, 40, and 60 μM CORM-319 and 60 μM iCORM-319 were compared to saturated MbCO (Figure 5.9 B).

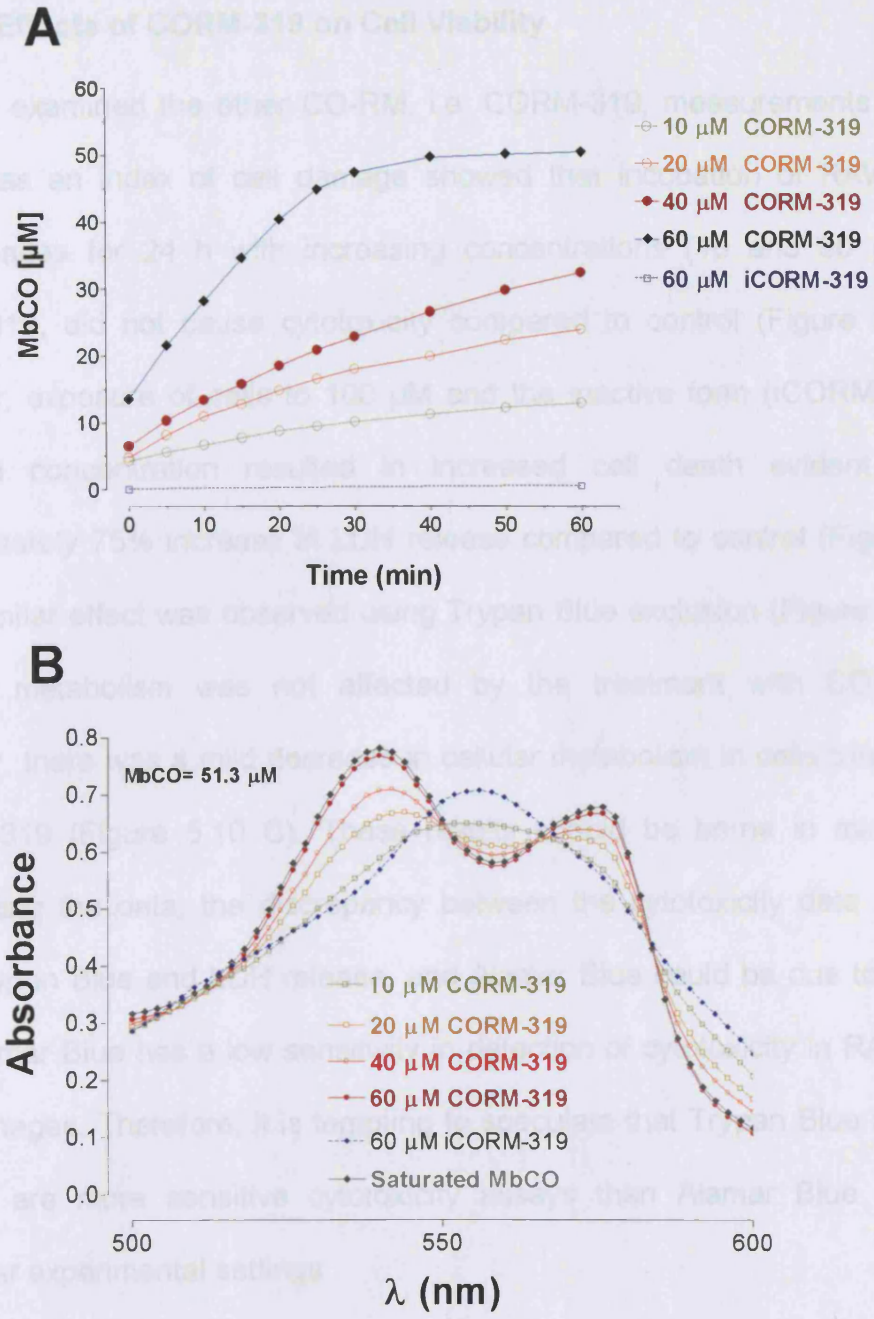



**Figure 5.9: Detection of CO released from CORM-319 in DMEM**

(A) Time course of CO released from 10, 20, 40, and 60 μM CORM-319 after incubation in DMEM medium (pH=7.4) at 37 ºC. The amount of CO released was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin (MbCO). (B) Spectra of MbCO formation at 60 min after addition of CORM-319 to myoglobin solution. Each line represents the mean ± SEM of 2 independent experiments. The rate of CO release was calculated from the fitted curves. iCORM-319, which does not release CO, was used as negative control.

### 5.5.10 Effects of CORM-319 on Cell Viability

We next examined the other CO-RM, i.e. CORM-319, measurements of LDH release as an index of cell damage showed that incubation of RAW 264.7 macrophages for 24 h with increasing concentrations (10 and 50 μM ) of CORM-319, did not cause cytotoxicity compared to control (Figure 5.10 A). However, exposure of cells to 100 μM and the inactive form (iCORM-319) at 100 μM concentration resulted in increased cell death evident by an approximately 75% increase in LDH release compared to control (Figure 5.10 A). A similar effect was observed using Trypan Blue exclusion (Figure 5.10 B). Cellular metabolism was not affected by the treatment with CORM-319; however, there was a mild decrease in cellular metabolism in cells treated with iCORM-319 (Figure 5.10 C). These results should be borne in mind when interpreting the data, the discrepancy between the cytotoxicity data obtained from Trypan Blue and LDH release, and Alamar Blue could be due to the fact that Alamar Blue has a low sensitivity in detection of cytotoxicity in RAW 2647 macrophages. Therefore, it is tempting to speculate that Trypan Blue and LDH release are more sensitive cytotoxicity assays than Alamar Blue in these particular experimental settings.

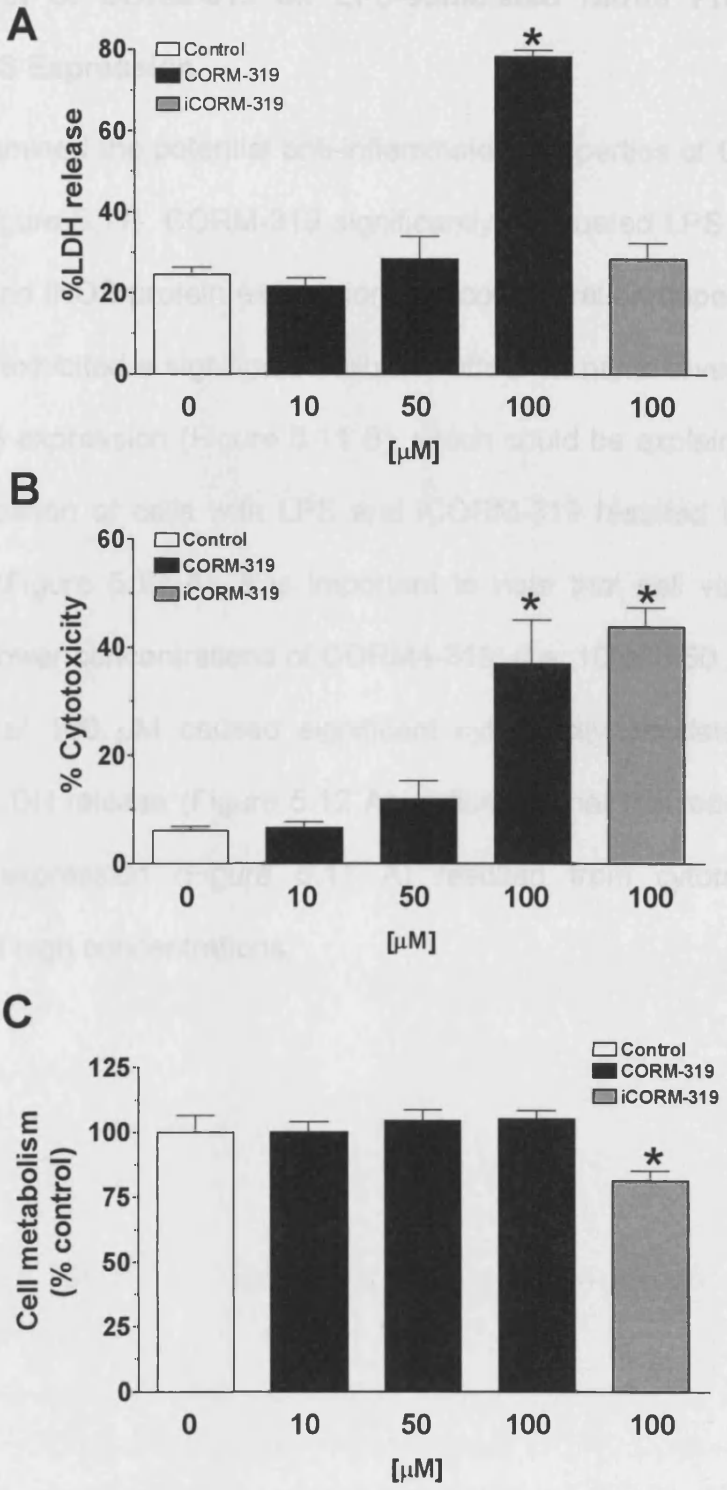



**Figure 5.10: Viability of cells exposed to CORM-319**

RAW 264.7 macrophages were exposed for 24 h to CORM-319 (10-100 μM) or iCORM-319 and cell viability was determined using LDH release (A) Trypan Blue exclusion (B) and Alamar Blue (C). Bars represent the mean ± S.E.M. of 6 independent experiments, * $P < 0.001$ vs. control.

### 5.5.11 Effect of CORM-319 on LPS-stimulated Nitrite Production and iNOS Expression

We then examined the potential anti-inflammatory properties of CORM-319. As shown in (Figure 5.11), CORM-319 significantly attenuated LPS-induced nitrite production and iNOS protein expression in a concentration-dependent manner; iCORM-319 exhibited a significant inhibitory effect on nitrite levels (Figure 5.11 A) and iNOS expression (Figure 5.11 B), which could be explained by the fact that co-incubation of cells with LPS and iCORM-319 resulted in a significant cytotoxicity (Figure 5.12 A). It is important to note that cell viability was not affected by lower concentrations of CORM4-319; (i.e. 10 and 50 μM). However, CORM-319 at 100 μM caused significant cytotoxicity as detected by 45% increase in LDH release (Figure 5.12 A), indicating that the reduction in nitrite and iNOS expression (Figure 5.11 A) resulted from cytotoxicity of this compound at high concentrations.

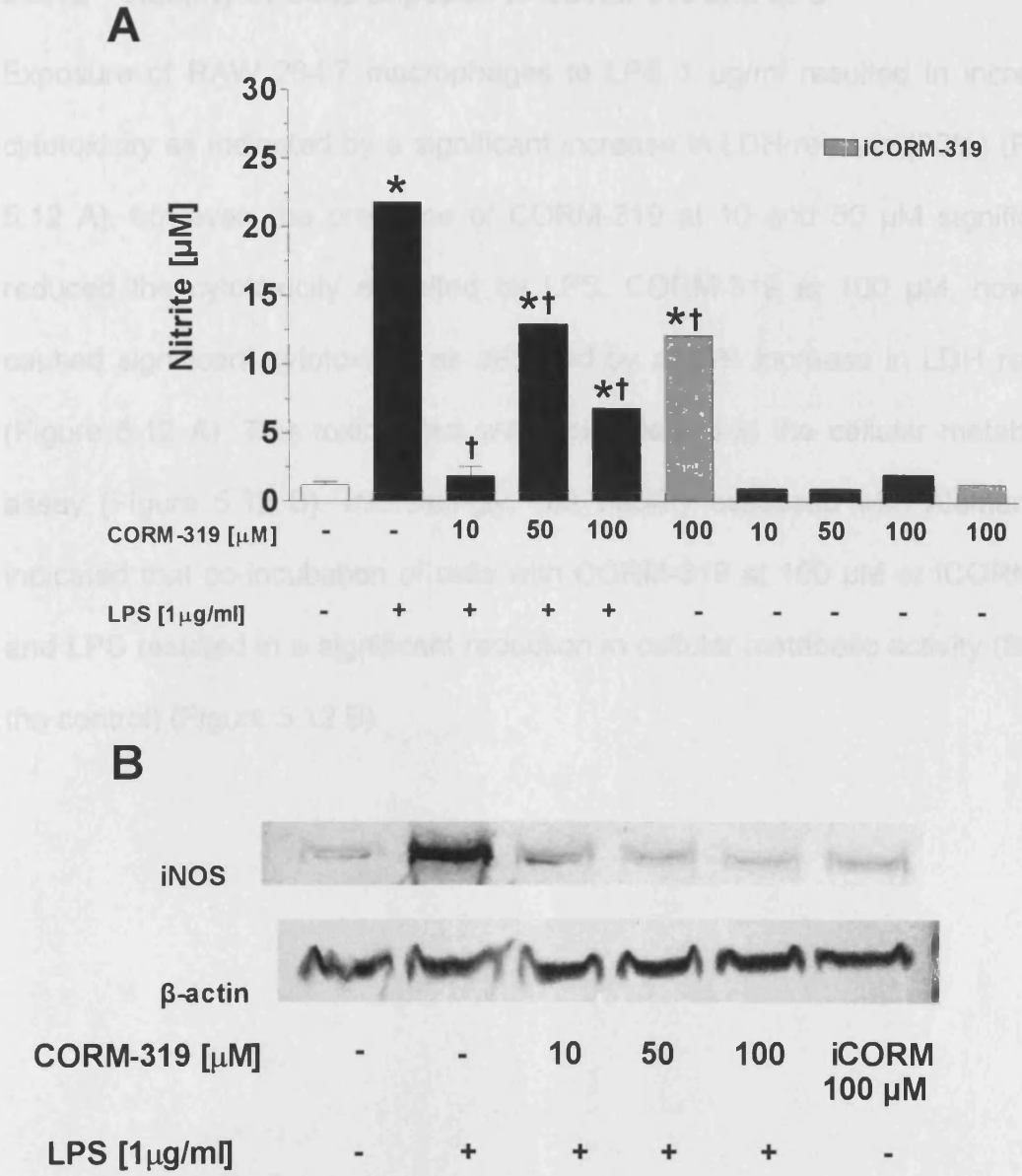



**Figure 5.11: CORM-319 modulates LPS-stimulated nitrite production and iNOS expression**.

RAW264.7 macrophages were exposed to various concentrations (10-100 μM) of CORM-319 or iCORM-319 (100 μM) in the presence of LPS 1 μg/ml for 24 h, nitrite production (A) and iNOS expression (B) were determined at 24 h. Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS. Western blot is a representative of three independent experiments. β-actin was used as an internal control for equal loading.

### 5.5.12 Viability of Cells Exposed to CORM-319 and LPS

Exposure of RAW 264.7 macrophages to LPS 1 μg/ml resulted in increased cytotoxicity as indicated by a significant increase in LDH release (30%) (Figure 5.12 A), however, the presence of CORM-319 at 10 and 50 μM significantly reduced the cytotoxicity exhibited by LPS. CORM-319 at 100 μM, however, caused significant cytotoxicity as detected by a 45% increase in LDH release (Figure 5.12 A). This toxic effect was not reflected in the cellular metabolism assay (Figure 5.12 B). Interestingly, cell viability assessed with Alamar Blue indicated that co-incubation of cells with CORM-319 at 100 μM or iCORM-319 and LPS resulted in a significant reduction in cellular metabolic activity (80% of the control) (Figure 5.12 B).

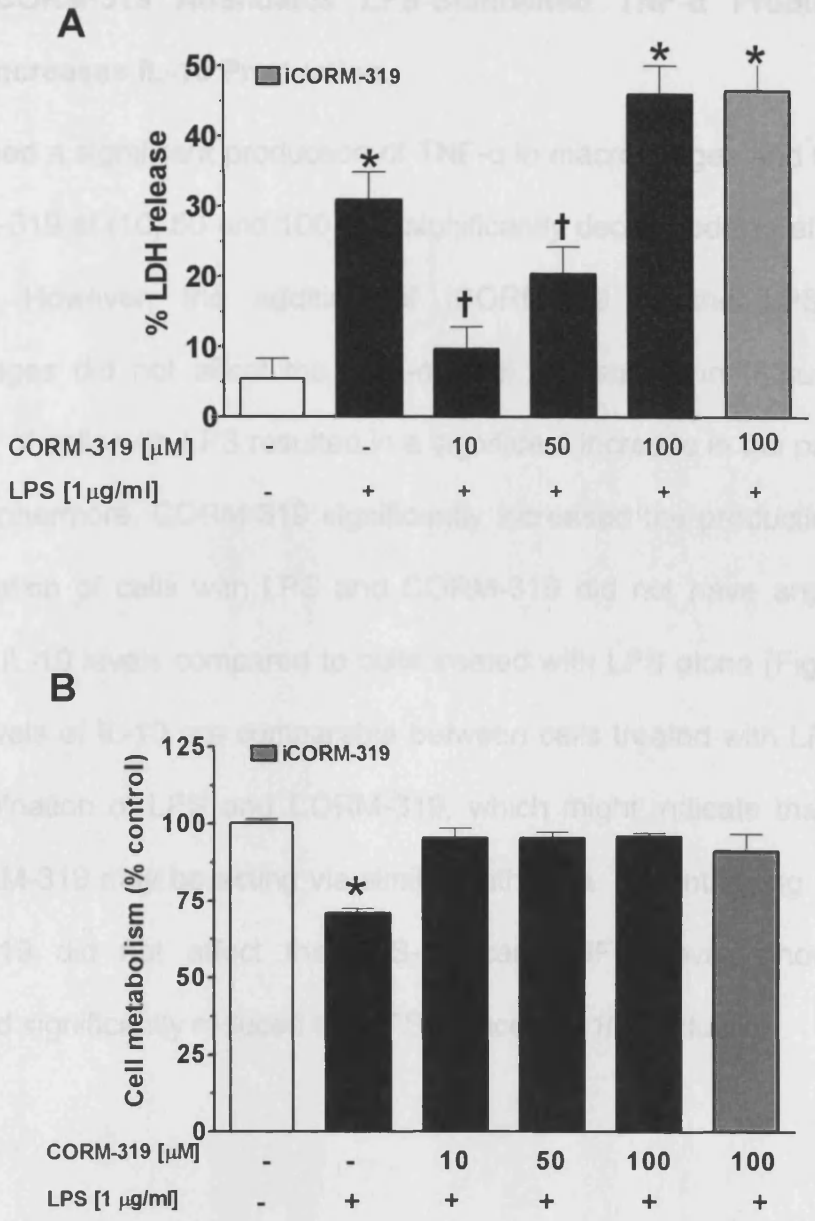



**Figure 5.12: Viability data in macrophages exposed to CORM-319 and LPS**

RAW264.7 macrophages were exposed to various concentrations (10-100 μM) of CORM-319 or iCORM-319 (100 μM) in the presence of LPS 1 μg/ml for 24 h. Cell viablity was then determined using LDH release (A) and Alamar Blue (B). Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control. † $P < 0.001$ vs. LPS. Western Blot is a representative of three independent experiments. β-actin was used as an internal control for equal loading.

### 5.5.13 CORM-319 Attenuates LPS-Stimulated TNF-α Production and Increases IL-10 Production.

LPS caused a significant production of TNF-α in macrophages and the addition of CORM-319 at (10, 50 and 100 μM) significantly decreased this effect (Figure 5.13 A). However, the addition of iCORM-319 to the LPS-stimulated macrophages did not affect the TNF-α level. As shown in (Figure 5.13 B), treatment of cells with LPS resulted in a significant increase in the production of IL-10. Furthermore, CORM-319 significantly increased the production of IL-10. Co-incubation of cells with LPS and CORM-319 did not have any significant effect on IL-10 levels compared to cells treated with LPS alone (Figure 5.13 B) as the levels of IL-10 are comparable between cells treated with LPS alone or the combination of LPS and CORM-319, which might indicate that both LPS and CORM-319 may be acting via similar pathways. It is intriguing to note that iCORM-319 did not affect the LPS-induced TNF-α levels, however, this compound significantly reduced the LPS-induced IL-10 production.

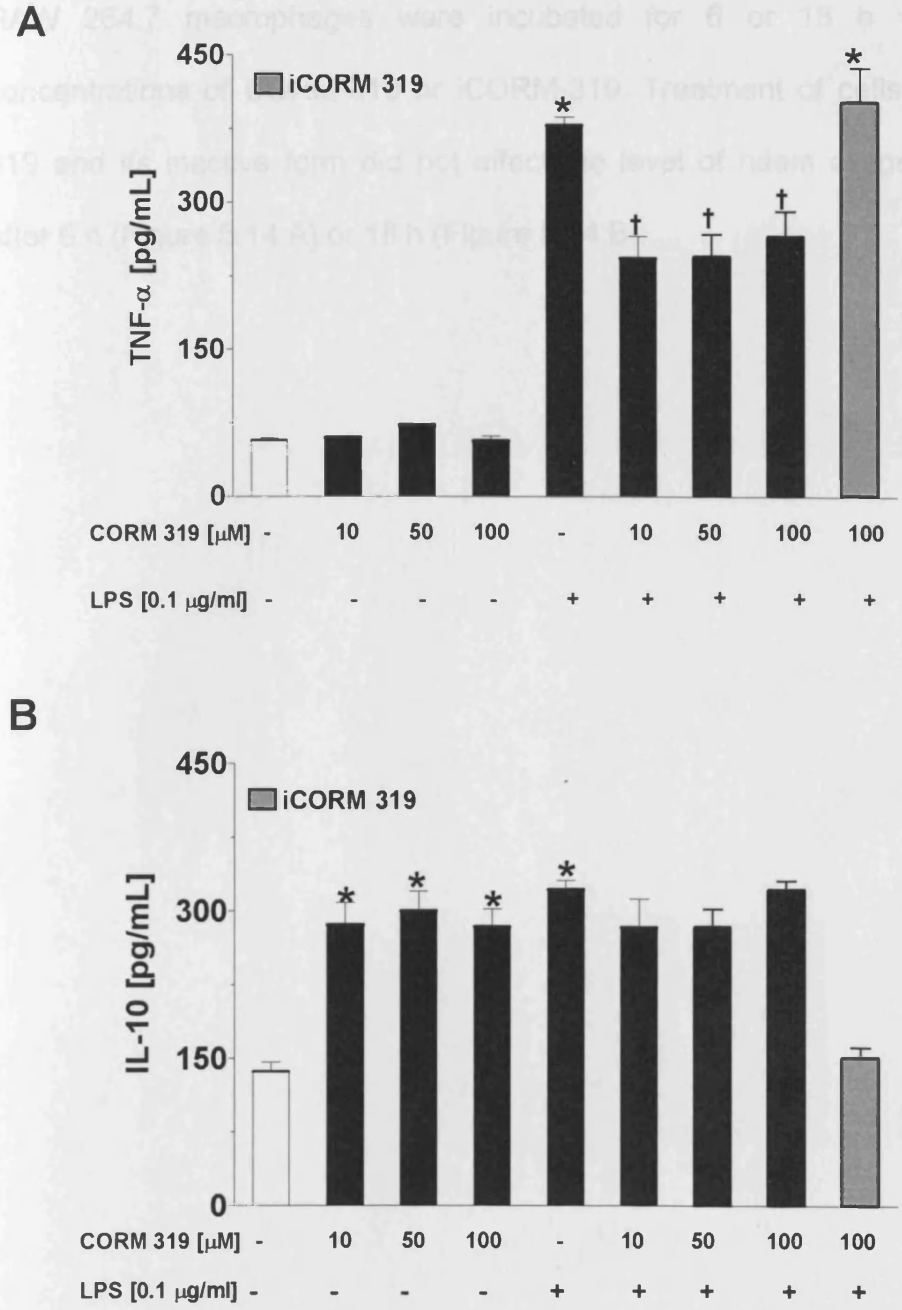



**Figure 5.13: CORM-319 attenuates LPS-stimulated TNF-α production and increases the production of IL-10.**

RAW264.7 macrophages were exposed to LPS (0.1 μg/ml) for 24 h in the presence or absence of CORM-319 (10-100 μM) or iCORM-319. TNF-α production (A) and IL-10 (B) were then determined. Bars represent the mean ± S.E.M. of 6 independent experiments, * $P < 0.001$ vs. control, † < 0.05 vs. LPS.

### 5.5.14 The Effect of CORM-319 on Haem Oxygenase Activity

RAW 264.7 macrophages were incubated for 6 or 18 h with different concentrations of CORM-319 or iCORM-319. Treatment of cells with CORM-319 and its inactive form did not affect the level of haem oxygenase activity after 6 h (Figure 5.14 A) or 18 h (Figure 5.14 B).

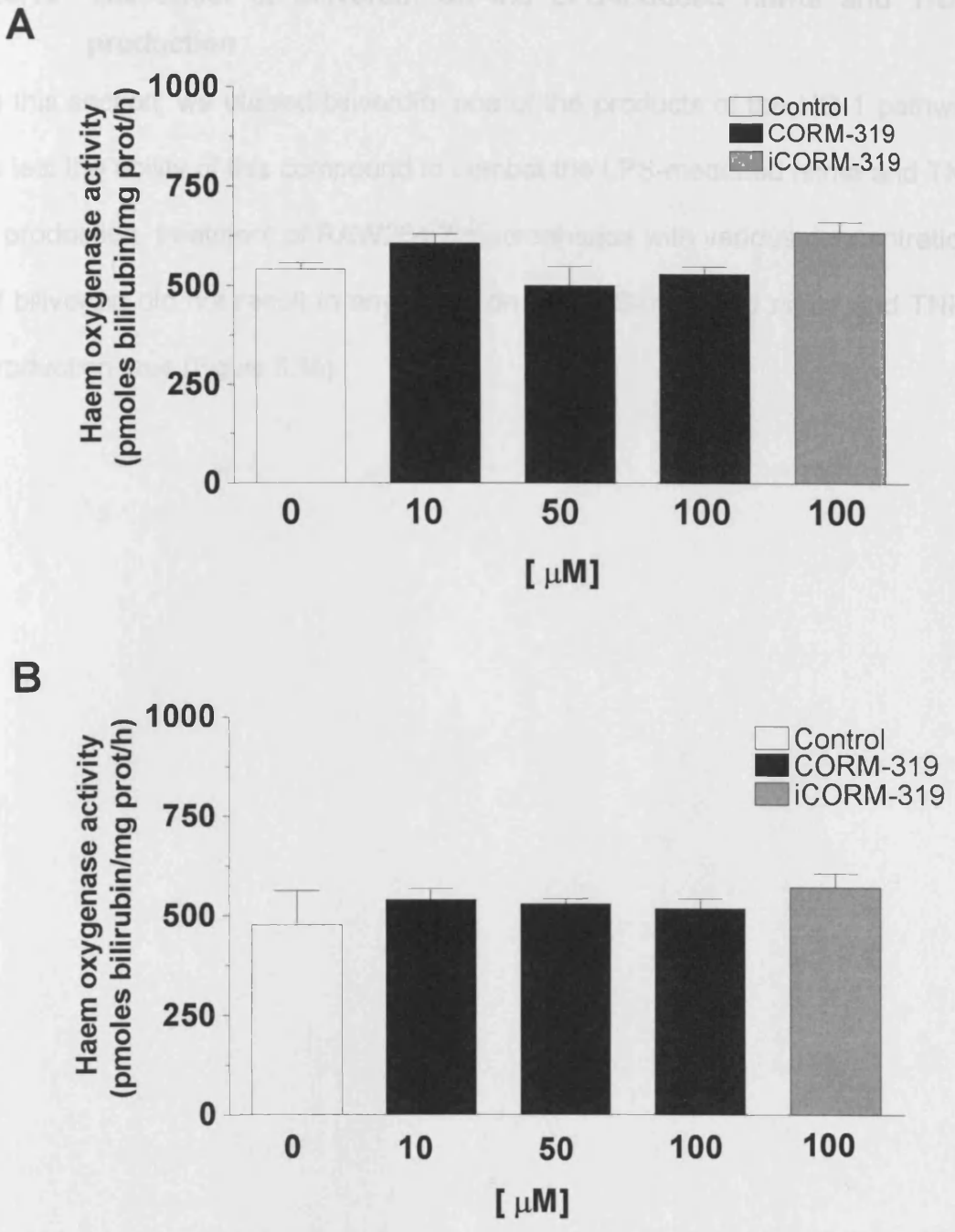



**Figure 5.14: The effect of CORM-319 on haem oxygenase activity in RAW264.7 macrophages**.

RAW 264.7 macrophages were exposed to various concentrations (10-100 μM) of CORM-319 or iCORM-319 for 6 h (A) or 18 h (B). Haem oxygenase activity was then determined at the end of the incubation. Cells treated with medium alone represented control (0). Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. 0 μM.

### 5.5.15 The effect of biliverdin on the LPS-induced nitrite and TNF-α production

In this section, we utilised biliverdin, one of the products of the HO-1 pathway, to test the ability of this compound to combat the LPS-mediated nitrite and TNF-α production, treatment of RAW264.7 macrophages with various concentrations of biliverdin did not result in any effect on the LPS-mediated nitrite and TNF-α production see (Figure 5.15).

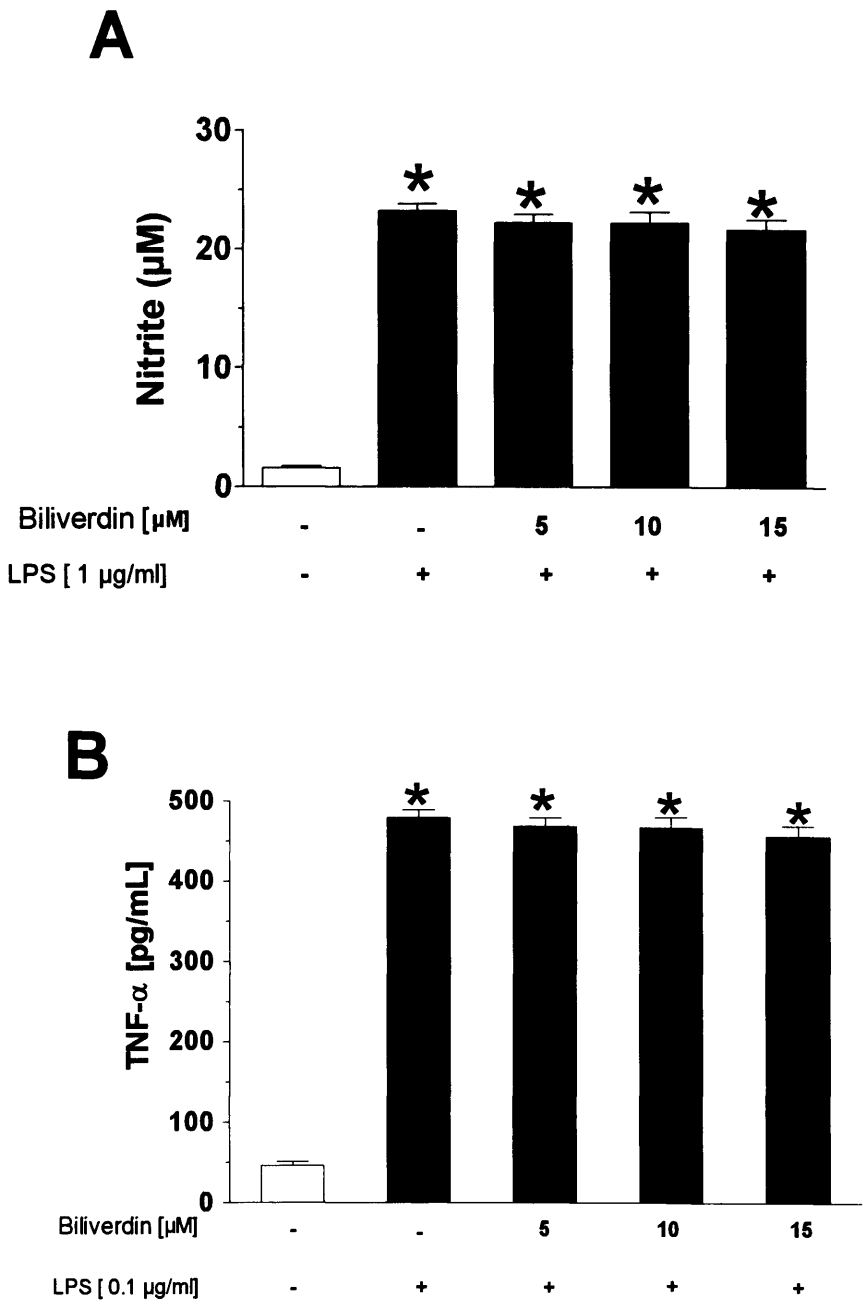



**Figure 5.15 The effect of biliverdin on the LPS-induced nitrite and TNF-α production**

(A) RAW264.7 macrophages were exposed to various concentrations (5-15 µM) of biliverdin in the presence of LPS 1 µg/ml for 24 h. Nitrite production was then determined. (B) RAW264.7 macrophages were exposed to LPS (0.1 µg/ml) for 24 h in the presence or absence of biliverdin (5-15 µM) TNF-α production were then determined. Bars represent the mean ± S.E.M. of 6 independent experiments, * $P < 0.001$ vs. control.

## 5.6 Discussion

There is a growing interest in the role of CO in cytoprotection, CO administered as a gas was shown to exhibit protective effects in I/R injury in rat intestinal grafts (Nakao et al., 2003), in lung hyperoxic injury (Otterbein et al., 1999) and suppressed the acute hypertensive response *in vivo* (Motterlini et al., 1998). In many experimental approaches, the inhalation or administration of exogenous CO gas in therapeutic applications showed beneficial results (Otterbein et al., 1999). However, these methods of delivering CO have limitations, including handling and storage of CO gas (Motterlini et al., 2005a). The need for a more practical and reliable method of delivering CO into the tissue in a controllable fashion lead to the development of new compounds, which have the ability to release CO in biological milieu (Motterlini et al., 2005b). Recently, our group developed a group of transitional metal carbonyls which have the intrinsic ability to liberate CO in biological systems (Clark et al., 2003). These groups have been identified generically as CO-releasing molecules (CO-RMs). Our initial studies showed that these compounds have important beneficial effects in biological systems, with versatile chemical properties that could facilitate the delivery of CO into the biological milieu (Foresti, 2004) and could be considered as a potential therapeutic approach in many pathological states (Clark et al., 2003). CO-RMs have similar beneficial pharmacological effects characteristics of CO gas, which include: vaso-relaxation of pre-contracted isolated aortic rings (Motterlini, 2005) (Foresti, 2004), CO-RMs attenuated the LPS-induced inflammatory response in RAW 264.7 murine macrophages (Sawle et al., 2005) and reduced the infarct size *in vivo* (Guo et al., 2004).

In the present study, two new water soluble CO-RMs (CORM-43 and CORM-319) were investigated in an *in vitro* model of inflammation using LPS-induced inflammation in RAW 264.7 murine macrophages. In the present study, we found that CORM-43 and CORM-319 exhibited inhibitory effects on the LPS-induced nitrite production and iNOS expression. Incubation of RAW 264.7 macrophages with CORM-43 for 24 h of did not cause cytotoxicity as measured by LDH release and Trypan Blue, however, iCORM-43 at 100 μM resulted in increased cytotoxicity evident by approximately 75% increase in LDH release and Trypan Blue, while cellular metabolism was normal. It is worth mentioning that the discrepancy in the results of the three assays should be borne in mind, the Alamar blue assay results should be considered with caution, as the results of the Alamar blue showed no cytotoxicity in cells treated with iCORM-43, while LDH release and trypan blue showed 75% cytotoxicity. This could be due to interference of iCORM-43 and the Alamar Blue dye. It is worth mentioning that the treatment of macrophages with the combination of LPS and either CORM-43 or CORM-319 did not result in any cytotoxicity, therefore, the reduction in the nitrite production and iNOS expression was solely due to the effects of these CO-RMs. However, co-incubation of CORM-319 at a concentration of 100 μM and LPS resulted in a significant cytotoxicity as measured by LDH release, and therefore, we conclude that the inhibitory effect of CORM-319 at 100 μM on nitrite and iNOS expression is due to cytotoxicity. These results are in disagreement with the findings obtained by our group previously, in which two other water soluble CO-RMs (CORM-3 and CORM-2) did not affect iNOS expression in murine macrophages (Sawle et al., 2005). It is interesting to note that different CO-RMs exert differential effects on iNOS expression, which might

be due to the different chemical structures of these compounds. To further elucidate the different aspects of the anti-inflammatory properties of CORM-43 and CORM-319, the effects of these compounds on the production of the pro-inflammatory (TNF-α) and the anti-inflammatory (IL-10) cytokines were studied. Our data showed that CORM-43 did not affect the LPS-induced TNF-α production, while at higher concentrations (50-100 μM) CORM-43 increased the production of this pro-inflammatory cytokine, it is possible that CORM-43 at higher concentrations acts as a pro-inflammatory agent. However, it is interesting to note that both CORM-43 and CORM-319 caused a significant increase in the production of IL-10 in un-stimulated cells, in LPS-stimulated cells, CORM-43 and CORM-319 did not have any significant effect on the production of IL-10. This is in a strong support of the anti-inflammatory properties of CO-RMs (Sawle et al., 2005). It is tempting to speculate that the CO released from both CORM-43 and CORM-319 exert their anti-inflammatory properties by up-regulation of anti-inflammatory cytokine, while not affecting the production of the pro-inflammatory cytokines, it has been shown that CO augmented the production of the ant-inflammatory IL-10 and mediated anti-inflammatory effects of IL-10 in mice (Lee and Chau, 2002).

The NF-KB family of transcription factors controls the expression of many genes, including genes encoding cytokines (Sarady et al., 2004). We speculated that CO exerts its anti-inflammatory effects by inhibiting the LPS-mediated activation of NF-KB pathway (Sarady et al., 2002). We therefore investigated the effect of CO released from CORM-43 on the activation of NF-KB. Our data demonstrated that CORM-43 did not have any significant effect on LPS-mediated translocation of the p65 of NF-KB to the nucleus. Therefore, it is

plausible to conclude that this CORM-43 does not exert inhibitory effects on the NF-KB pathway, and probably activates or deactivates other cellular pathways that mediate its inhibitory effects on the cytokines and on nitrite production. However, one cannot make a conclusion without undertaking further detailed studies to elucidate the effect of CORM-43 on the activation of NF-KB, such as measuring the activation of NF-KB using EMSA as well as the assessment of the integrity of IKB. Our data is in disagreement with data in the literature that showed that CO inhibited the activation of NF-KB (Sarady et al., 2002). Furthermore, our data showed that CORM-43 results in the activation of the AKT pathway, our data is in agreement with previous data in the literature which showed that CO gas targets the AKT pathway (Fujimoto et al., 2004). We then investigated the mechanisms by which CORM-43 and CORM-319 exert their anti-inflammatory effects; one possible pathway is the haem oxygenase system. It was shown that other CO-RMs are potent inducers of haem oxygenase activity and HO-1 expression (Sawle et al., 2005). Treatment of cells with CORM-43 resulted in a minor (insignificant) increase in haem oxygenase activity after 6 h and to a lesser extent after 18 h incubation. Furthermore, the effect of iCORM-43 on haem oxygenase activity was comparable to the effect of CORM-43, and therefore, this effect is not CO-dependent. In addition, treatment of cells with iCORM-43 resulted in cytotoxicity, which indicates that the insignificant effect of iCORM-43 on haem oxygenase activity might be due the effect on the cells. We conclude that CORM-43 and CORM-319 are targeting cellular pathways that do not involve the haem oxygenase system. Our results showed that biliverdin, one of the products of the HO-1 pathway, was unable to reduce the LPS-mediated nitrite and TNF-α production, treatment of RAW264.7

macrophages with various concentrations of biliverdin did not result in any effect on the LPS-mediated nitrite and TNF-α production. This data is in agreement with data provided by our group (Swale et al., 2005).

In conclusion, the present study identified two new water soluble CO-RMs, and explored their anti-inflammatory effects, furthermore, it supports the anti-inflammatory action of CO-RMs in an *in vitro* model of LPS-mediated inflammation and supports the further testing of CO-RMs.

# 6 CURCUMIN ATTENUATES THE OXIDANT-DAMAGE STIMULATED BY $H_2O_2$ IN HUMAN CARDIAC MYOBLASTS

## 6.1 Introduction

Curcumin is a naturally-occurring yellow pigment presents in the plant *Curcuma longa* (turmeric) (Ammon and Wahl, 1991). The significance of this compound in health and nutrition has changed considerably since the discovery of the antioxidant properties of naturally occurring phenolic compounds (Balasubramanyam et al., 2003). The dried rhizome of *C. longa* (used as a spice, food preservative and a colouring agent) is a rich source of beneficial phenolic compounds; the curcuminoids, which are a group of phenolics present in turmeric (Chainani-Wu, 2003). Three major curcuminoids have been isolated from turmeric namely: curcumin (diferuloylmethane) which makes up approximately 90% of the curcuminoids content, followed by demethoxycurcumin, and bisdemethoxycurcumin (Ruby et al., 1995). In this study we have tested curcumin (

), which possesses intrinsic anti-inflammatory, antioxidant and anti-tumour actions (Song et al., 2001) (Lim et al., 2001) (Huang et al., 1991) (Menon et al., 1999).

It has been suggested that oxidative stress play a major role in the pathogenesis of cardiovascular diseases (Hamilton et al., 2004). Oxidative stress is the result of excessive production of ROS and or depletion of intracellular antioxidant defenses, leading to an imbalance in the redox status of the cell (Miquel et al., 2002). Due largely to the detrimental nature of ROS and electrophilic species, mammalian cells have evolved a number of anti- oxidative

and phase II detoxifying enzymes to protect against oxidative and electrophilic cell damage (Talalay, 2005). In recent years, much attention has been focused on HO-1, one of the phase II enzyme responses (Owuor and Kong, 2002). It has been suggested that it functions primarily as an effective system to counteract oxidative stress (Maines, 1997). HO-1 is the rate-limiting enzyme in the conversion of haem into biliverdin, releasing free iron and carbon monoxide (Maines, 1997). Biliverdin is rapidly metabolized to bilirubin, which is a potent antioxidant (Otterbein and Choi, 2000). The proposed antioxidant role for HO-1 is based on some crucial experimental observations: HO-1 gene expression is extremely sensitive to up-regulation by oxidative stress in a variety of mammalian tissues (Ryter et al., 2002), induction of HO-1 protein protects tissues against oxidant-mediated injury (Llesuy and Tomaro, 1994), and HO-1 knockout mice exhibited reduced stress defenses when exposed to oxidative challenge (Agarwal and Nick, 2000). Since biliverdin and bilirubin have been shown to possess potent antioxidant properties (Ryter et al., 2002) and up-regulation of HO-1 is usually accompanied by increased levels of ferritin, a protein which sequesters intracellular catalytic iron (Balla et al., 1992), HO-1 appears to be an excellent candidate for cytoprotection. Therefore, HO-1 can be regarded as a potential therapeutic target in a variety of oxidant-mediated diseases. In this respect, the search for novel and more potent inducers of this pathway will facilitate the development of pharmacological strategies to increase the intrinsic capacity of cells to maximize HO-1 expression and, ultimately, cytoprotection (Motterlini et al., 2000b).

There has been mounting evidence in the literature which showed that curcumin is a strong inducer of HO-1 in various cell types. It was demonstrated that

curcumin induces HO-1 and protects endothelial cells, astrocytes and renal epithelial cells (Scapagnini et al., 2002) (Balogun et al., 2003a) against oxidative stress. It is suggested that some beneficial effects of curcumin might be mediated by its inherent ability to increase HO-1 and possibly other intracellular protective pathways such as activation of the endogenous detoxifying enzymes (e.g. phase II enzymes) for (Motterlini et al., 2000b). Recently, curcumin has been shown to exhibit anti-inflammatory (Chainani-Wu, 2003), and anti-atherogenic activities due to its ability to scavenge superoxide anions (Sugiyama, 1996). In the present study, we aimed to investigate the protective effect of curcumin against $H_2O_2$-induced cell damage in human cardiac myoblasts (Girardi cells). In this study we attempted to explore the possible mechanisms underlying the antioxidant effects of curcumin, with focus on the role of HO-1.

## 6.2 Objectives

To study the effects of curcumin on haem oxygenase activity and HO-1 protein expression in Girardi cells, in addition to examining the effects of curcumin on $H_2O_2$-mediated oxidative stress in Girardi cells.

## 6.3 Materials and Methods

### 6.3.1 Reagents

Curcumin and all other reagents were purchased from Sigma. Haemin and Tin Protoporphyrin were obtained from Porphyrin Products INC (Logan, Utah, USA). Alamar Blue reagent was obtained from Serotec; (Oxford, UK).

### 6.3.2 Cell Culture

Girardi (Human cardiac myoblasts) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK) and cultured in Dulbecco's modified Eagle's medium containing, 3.5 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and supplemented with 10% FBS, in addition to 1% Non-essential amino acids. Cells were grown in 75-$cm^2$ flasks and maintained at 37°C in a humidified atmosphere of air and 5% $CO_2$. Confluent cells were exposed to various reagents, haem oxygenase activity, HO-1 protein expression and cell viability assays were employed after each treatment.

### 6.3.3 Experimental Protocol

Confluent cells were exposed to curcumin (15 μM) for 2, 4, and 6 h, haem oxygenase activity was then determined at the end of the incubation. Haem oxygenase activity and HO-1 protein expression were also measured in cells exposed for 6 to various concentrations (5, 15 and 30 μM) of curcumin or haemin (5-20 μM). Cell viability was assessed using a metabolic assay (Alamar Blue) and LDH release after 24. To assess the range of cytotoxicity induced by

$H_2O_2$ in the cells, Girardi cells were treated with increasing concentrations of $H_2O_2$ (0.75, 1.5, and 3 mM) for 2, 4 and 6 h; cell viability was then assessed using Alamar Blue. The potential anti-oxidant effects of curcumin was also examined; for this purpose, cells were pre-treated with this compound for 6 h, the culture medium was then removed and cells were then exposed for 2, 4 or 6 h to various concentrations (0.75, 1.5 and 3 mM) of $H_2O_2$. In a similar set of experiments, haemin (5 or 10 μM) was added to examine the possibility of synergism between haemin (a known inducer of HO-1) and curcumin. To assess the range of cytotoxicity induced by $H_2O_2$ in the cells, for this purpose, cells were treated with increasing concentrations of $H_2O_2$ (0.75, 1.5, and 3 mM) for 2, 4 and 6 h; cell viability was then assessed by measuring the LDH release. Similar experiments were performed using higher concentrations of $H_2O_2$ (3, 6 and 9 mM) for 2, 4 and 6 h. In order to examine the involvement of HO-1 in the anti-oxidant effects of curcumin, cells were pre-treated with of curcumin 15 μM for 6 h and then exposed to different concentrations of $H_2O_2$ (3, and 6 mM) for 6 h in the presence or absence of SnPPIX 10 μM. Viability was then determined using Alamar Blue assay and LDH release.

### 6.3.4 Cell Viability/Alamar Blue Assay

Cell viability was determined using an Alamar Blue assay kit, it was carried out as previously described in Section 2.3.1.

### 6.3.5 LDH Assay

Extracellular, i.e., released, LDH activity was measured using cytotoxicity detection kit (Roche) as previously described in section 2.3.2.

### 6.3.6 Trypan Blue Assay

Trypan Blue exclusion was performed as previously described in section 2.3.3.

### 6.3.7 Haem Oxygenase Activity Assay

Haem oxygenase activity was determined at the end of each treatment as described previously in Section 2.4.3.

### 6.3.8 Western Blot Analysis for HO-1

Cells were also analyzed for the determination of the protein expression for HO-1by Western immunoblot technique as previously described in Section 2.5.5.

## 6.4 Statistical Analysis

Statistical analysis was performed using one-way ANOVA combined with the Bonferroni test. Differences were considered to be significant at $P<0.05$.

## 6.5 Results

### 6.5.1 The Effect of Curcumin on Haem Oxygenase Activity and HO-1 Expression in Girardi Cells

It was reported previously that curcumin induces haem oxygenase activity and HO-1 expression in renal epithelial cells (Balogun et al., 2003b). In order to establish the effect of curcumin on haem oxygenase activity and HO-1 in Girardi cells, cells were exposed to curcumin (15 μM) for 2, 4 and 6 h. As shown in (Figure 6.1 A), treatment of cells with curcumin (15 μM) resulted in a time-dependent increase in haem oxygenase activity. When cells were exposed to increasing concentrations of curcumin (5-30 μM), a concentration-dependent increase in haem oxygenase activity HO-1 protein expression as shown in (Figure 6.1 B) was observed.

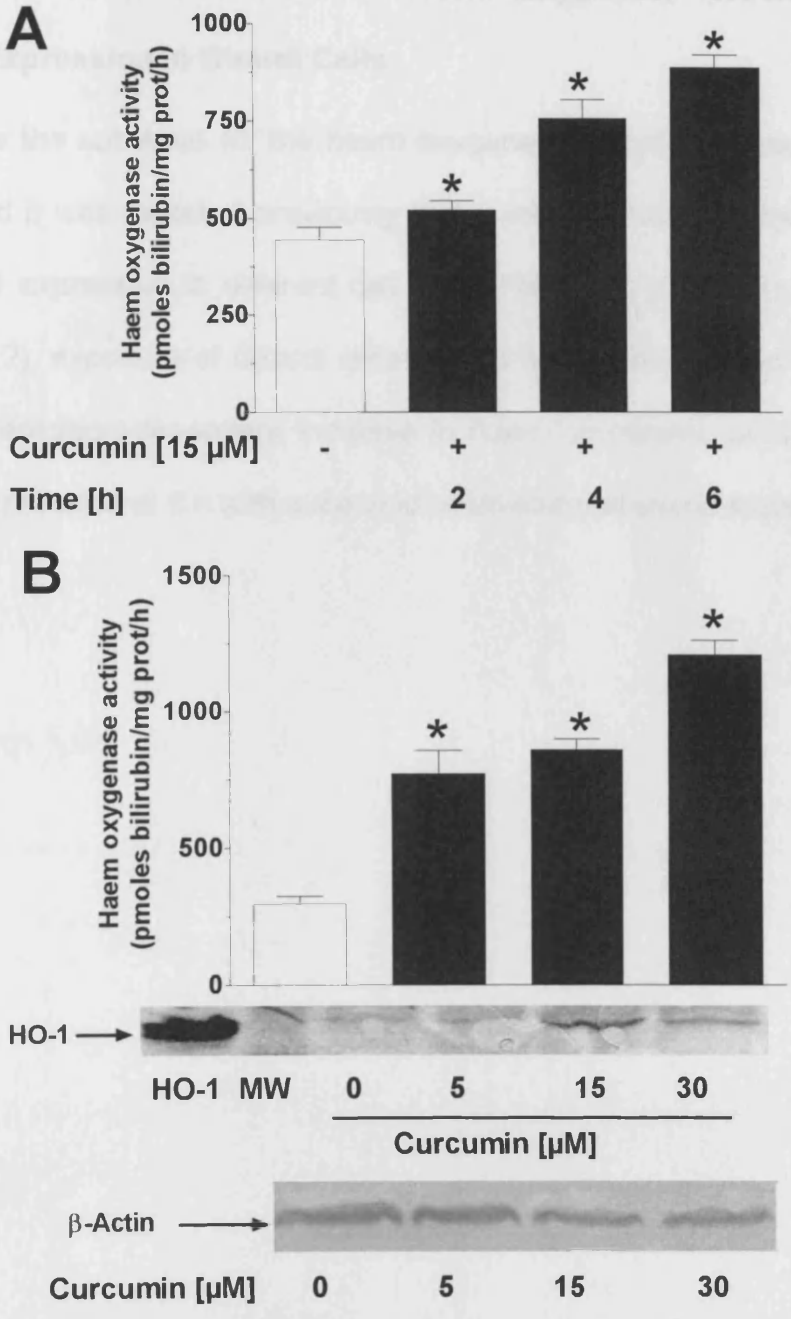



**Figure 6.1: The effect of curcumin on haem oxygenase activity and HO-1 expression in Girardi cells**

Girardi cells were exposed to curcumin 15 µM for 2, 4 and 6 h (A), and to curcumin (5-30 µM) for 6 h (B). Haem oxygenase activity and HO-1 protein expression were then determined at the end of the incubation. Cells treated with medium alone represented control (0). Western Blot is a representative of three independent experiments. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. control. β-actin was used as an internal control for equal loading. *HO-1:* positive control recombinant HO-1 protein, MW: molecular weight marker.

### 6.5.2 The Effect of Haemin on Haem Oxygenase Activity and HO-1 Expression in Girardi Cells

Haemin is the substrate for the haem oxygenase enzymatic reaction (Maines, 1988) and it was reported previously that it induces haem oxygenase activity and HO-1 expression in different cell lines (Foresti et al., 2003). As shown in (Figure 6.2), exposure of Girardi cells for 6 h to haemin resulted in a dramatic and concentration-dependent increase in haem oxygenase activity and HO-1 protein expression at 6 h with a maximal expression at concentration (20 μM).

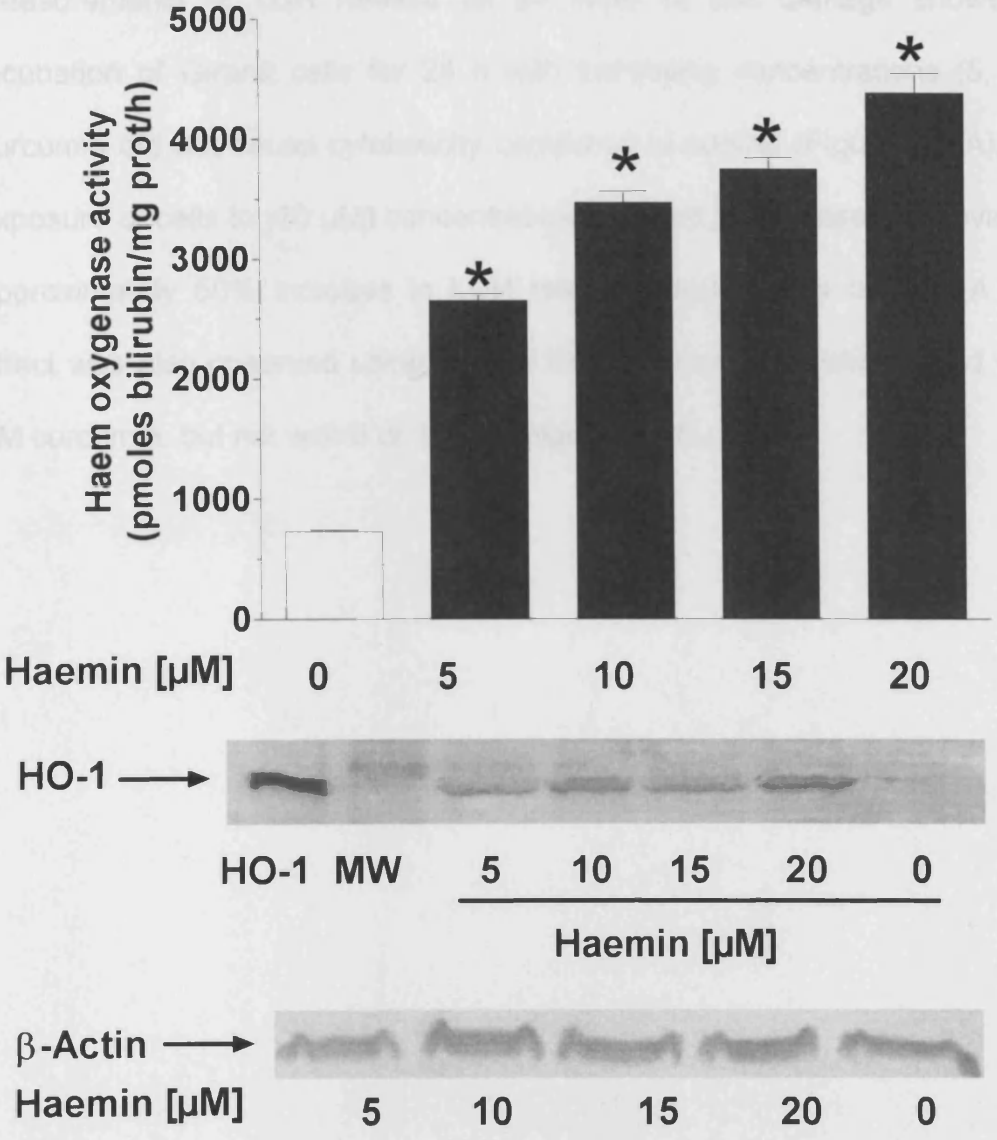



**Figure 6.2: The effect of haemin on haem oxygenase activity and HO-1 expression in Girardi cells**

Girardi cells were exposed to various concentrations (5-20 µM) of haemin for 6 h. Haem oxygenase activity and HO-1 protein expression were determined at the end of the incubation. Cells treated with medium alone represented control (0). Western Blot is a representative of three independent experiments. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group.* $P < 0.001$ vs. 0 µM. β-actin was used as an internal control for equal loading. *HO-1:* positive control recombinant HO-1 protein, MW: molecular weight marker.

### 6.5.3 Cytotoxicity in Cells Treated with Curcumin

Measurements of LDH release as an index of cell damage showed that incubation of Girardi cells for 24 h with increasing concentrations (5, 15) of curcumin did not cause cytotoxicity compared to control (Figure 6.3 A). While exposure of cells to (30 μM) concentration, resulted in increased cell evident by approximately 50% increase in LDH release compared to control. A similar effect was also observed using Trypan Blue exclusion, in cells treated with 30 μM curcumin, but not with 5 or 15 μM (Figure 6.3 B).

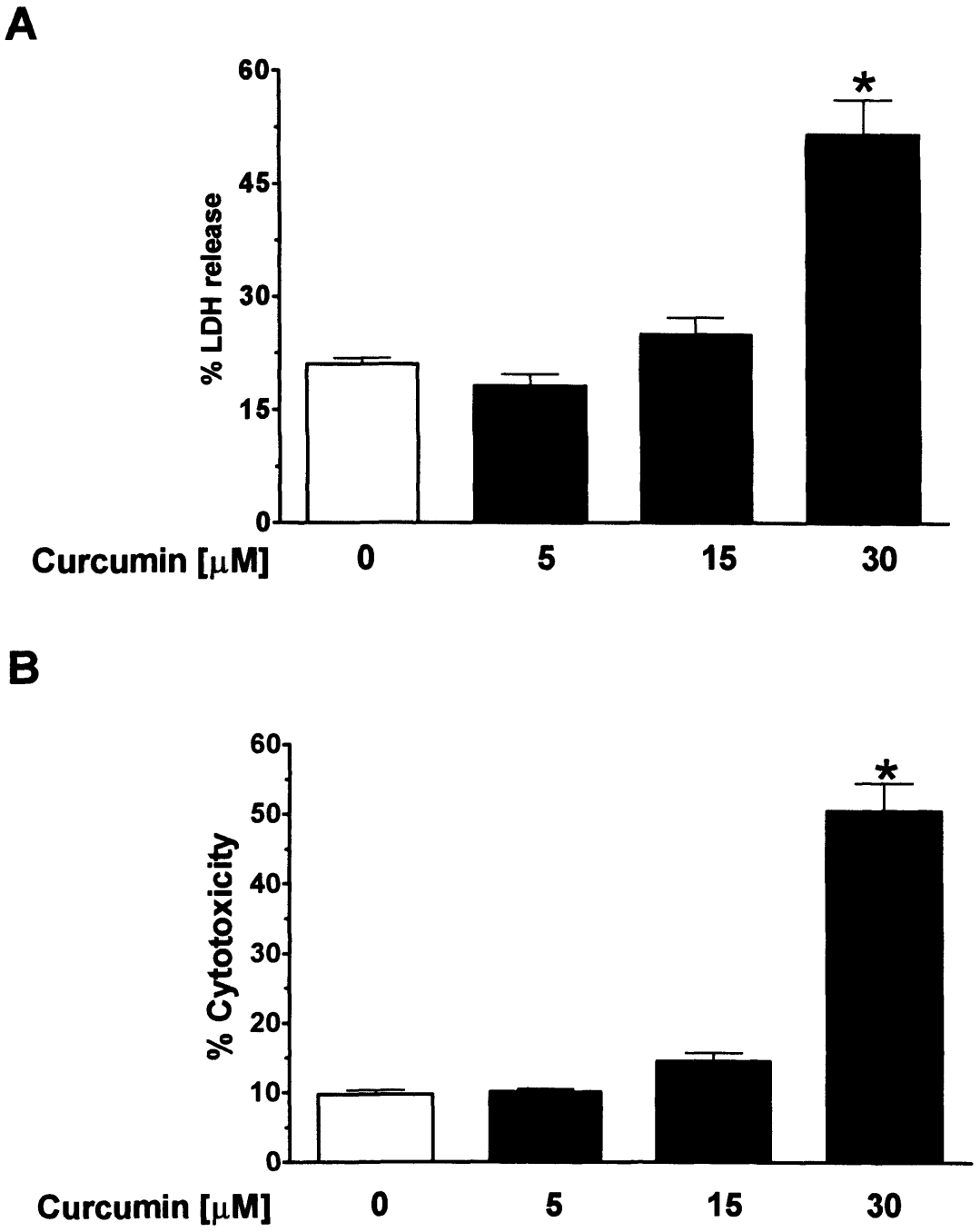



**Figure 6.3: Viability of cells exposed to curcumin.**

Girardi cells were exposed to for 24 h in the presence of curcumin (5-30 μM). Cell viability was determined using LDH release (A) and Trypan Blue exclusion (B). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control.

### 6.5.4 Effect of $H_2O_2$ on Cell Metabolism

Oxidative stress has been suggested to play a critical role in a number of cardiovascular pathologies, such as atherosclerosis and I/R injury, in which ROS including superoxide, $H_2O_2$ and hydroxyl radicals are found in the myocardium (Chen et al., 2000). Therefore, it is of importance to investigate the effects and the potential therapeutic approaches to the $H_2O_2$-mediated cardiac injury. First, we wanted to identify the cytotoxicity range of $H_2O_2$, for this purpose, Girardi cells were treated with increasing concentrations of $H_2O_2$ (0.75, 1.5, and 3 mM) for 2, 4 and 6 h (Figure 6.4). Exposure of cells to this protocol resulted in a concentration and time-dependent decline in cell metabolism (50%) (Figure 6.4 A), 40% (Figure 6.4 B) and 35% (Figure 6.4 C). Treatment of cells with $H_2O_2$ caused cytotoxicity evident by a significant decrease in cell metabolism in a concentration-dependent manner. Therefore, for subsequent protection experiments, we used this range of concentrations (1.5, and 3 mM) for 2 and 4 h-incubation.

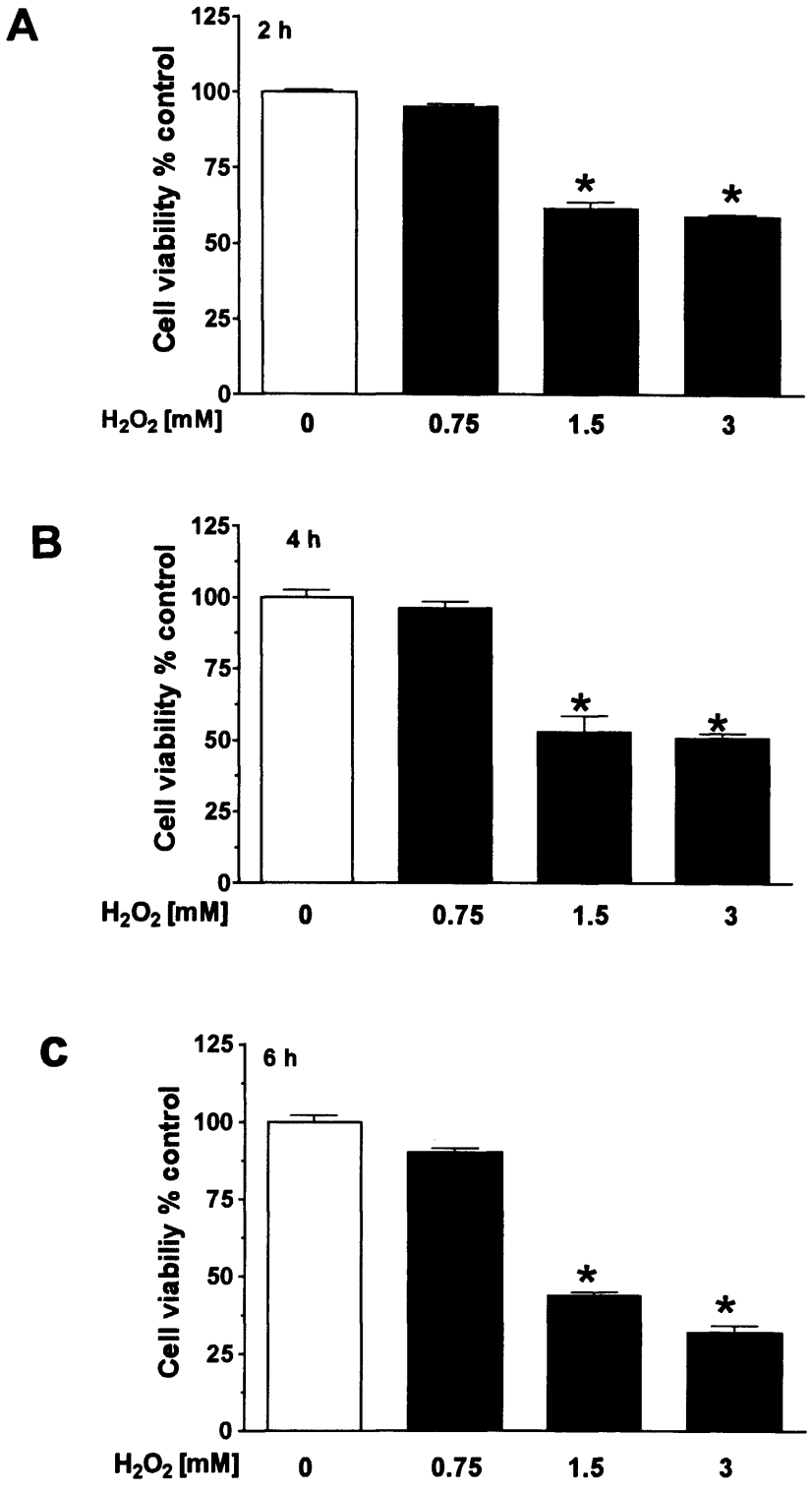



**Figure 6.4 : Effect of $H_2O_2$ on cell metabolism in Girardi cells**

Girardi cells were exposed to different concentrations of $H_2O_2$ (0.75, 1.5 and 3 mM) for 2 h (A) 4 h (B) or 6 h (C). Cell viability was then determined using Alamar Blue assay. Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control.

### 6.5.5 Effect of Curcumin on the $H_2O_2$–Mediated Oxidative Stress

We then tested if curcumin could counteract oxidative stress caused by treatment with $H_2O_2$. For this purpose, Girardi cells were initially pre-treated with curcumin (15 μM) for 6 h to allow HO-1 induction to take place, and then the growing medium was removed, and $H_2O_2$ (1.5, or 3 mM) was added to the cells grown in fresh medium. Treatment with increasing concentrations of $H_2O_2$ (1.5, or 3 mM) for 2, or 4 h resulted in a concentration-dependent decrease in cell metabolism 50% (Figure 6.5 A) and 40% (Figure 6.5 B) respectively, however, pre-treatment of cells with curcumin did not exert any significant effect on the $H_2O_2$-mediated cytotoxicity.

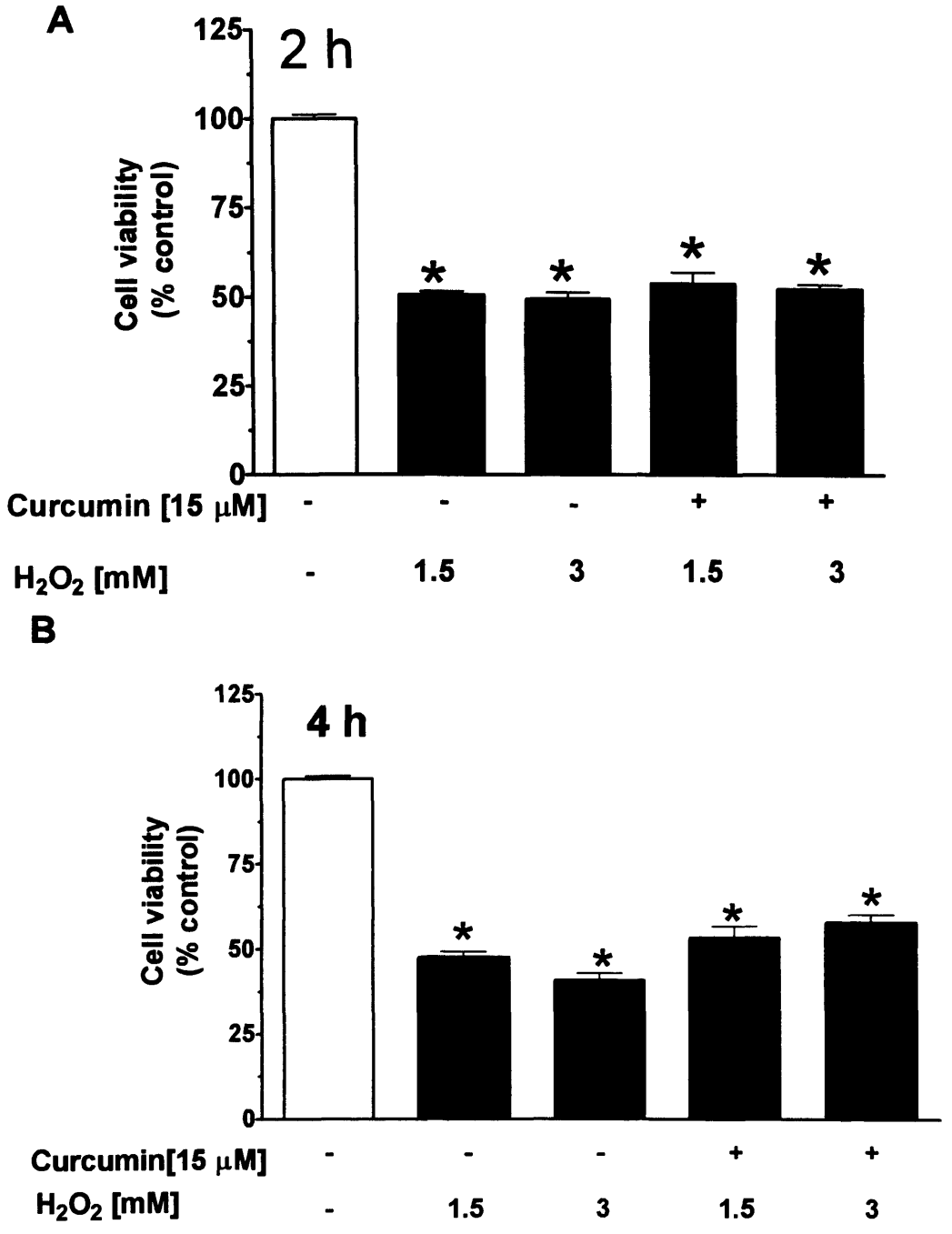



**Figure 6.5: Effect of curcumin on the $H_2O_2$–mediated oxidative stress**

Girardi cells were pre-treated with of curcumin 15 μM for 6 h, the culture medium was then removed and cells were exposed to different concentrations of $H_2O_2$ (1.5 and 3 mM) for 2 h (A) or 4 h (B). Cell viability was then determined using Alamar Blue assay. Bars represent the mean ± S.E.M. of 5-6 independent experiments. * $P < 0.001$ vs. control.

### 6.5.6 Effect of Curcumin and Haemin on the $H_2O_2$–Mediated Oxidative Stress

Since pre-treatment with curcumin (15 μM) did not attenuate the $H_2O_2$–mediated cytotoxicity, we tested if the combination of curcumin and haemin could counteract the cytotoxicity induced by $H_2O_2$. Having established that haemin at (5 μM) concentration has the ability to induce haem oxygenase activity and HO-1 expression in Girardi cells (Figure 6.2). Cells were initially pre-treated with curcumin (15 μM) and haemin (5 μM) for 6 h to allow for a possible synergism for HO-1 induction to take place. Treatment with increasing concentrations of $H_2O_2$ (1.5, and 3 mM) for 2 h or 4 h resulted in a concentration-dependent decline in cell metabolism (40% Figure 6.6 A) and 35% (Figure 6.6 B) respectively; pre-treatment of cells with curcumin and haemin however, did not have any significant effect on the $H_2O_2$-induced cytotoxicity (Figure 6.6).

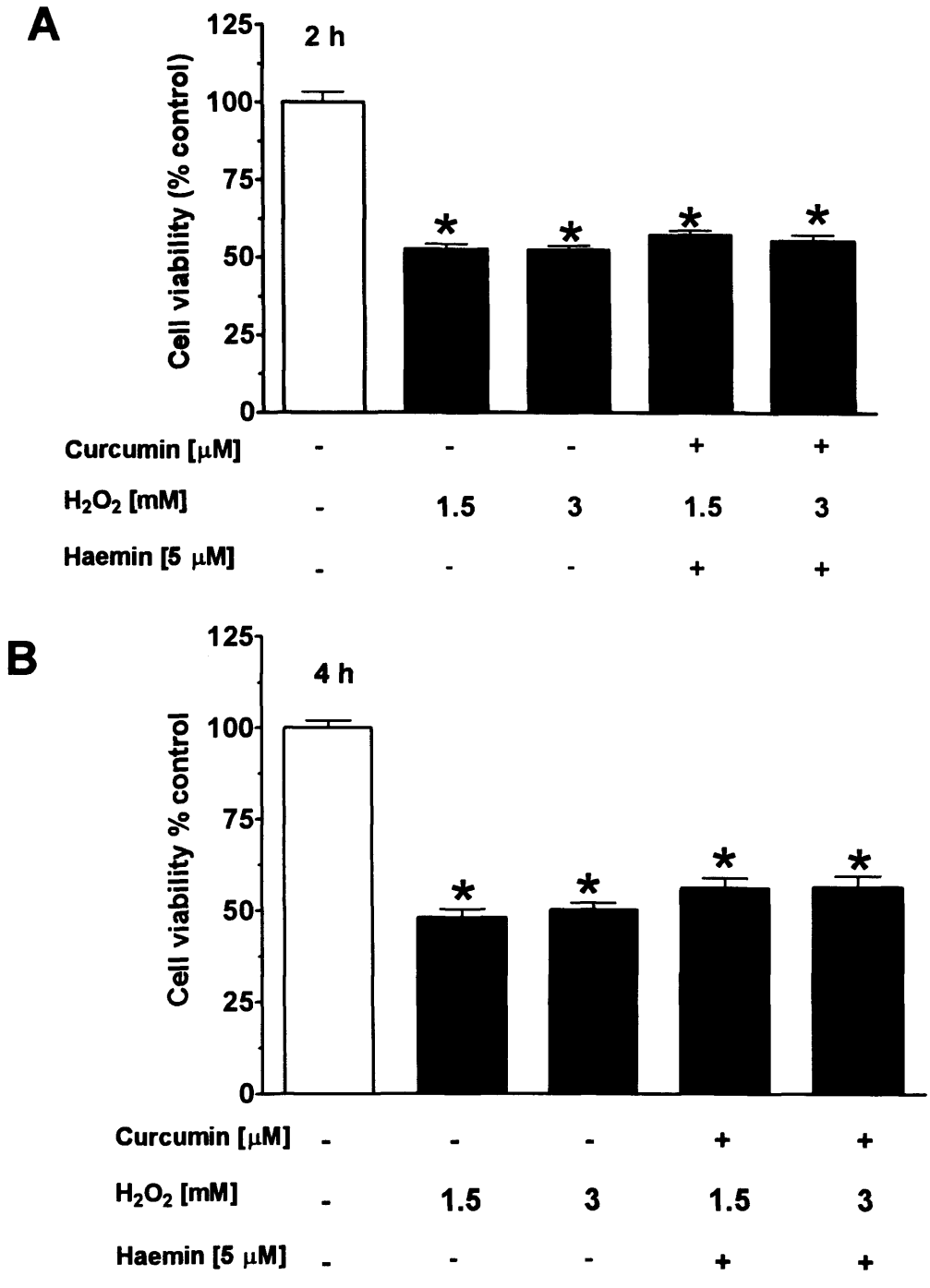



**Figure 6.6: Effect of curcumin and haemin (5 μM) on the $H_2O_2$–mediated oxidative stress**

Girardi cells were pre-treated with of curcumin 15 μM and haemin 5 μM for 6 h, the medium was then removed and cells were exposed to different concentrations of $H_2O_2$ (1.5 and 3 mM) for 2 h (A), 4 h (B). Viability was then determined using Alamar Blue assay. Bars represent the mean ± S.E.M. of 5-6 independent experiments. * $P < 0.001$ vs. control.

### 6.5.7 Effect of Curcumin and Haemin (10 μM) on the $H_2O_2$–Mediated Oxidative Stress

Since the combination of curcumin and haemin at (5 μM) was not protective against $H_2O_2$-mediated damage, we then tested if the combination of curcumin and haemin at a higher concentration (10 μM) could counteract the oxidative stress caused by treatment with $H_2O_2$. Girardi cells were initially pre-treated with curcumin (15 μM) and haemin (10 μM) for 6 h to allow HO-1 induction to take place; cells were then exposed to $H_2O_2$. Treatment with increasing concentrations of $H_2O_2$ (1.5, and 3 mM) for 2 h or 4 h resulted in a concentration-dependent decline in cell metabolism (40 and 35% respectively), pre-treatment of cells with curcumin (15 μM) and haemin (10 μM) did not have any significant effect on the $H_2O_2$-induced cytotoxicity after 2 h (Figure 6.7 A). However, in cells exposed to $H_2O_2$ for longer time point (4 h), the combination of curcumin and haemin exhibited a further decrease in cellular metabolism (Figure 6.7 B), which indicates that pre-exposure to curcumin and haemin at higher concentration (10 μM), rendered the cells more susceptible to cellular damage by $H_2O_2$.

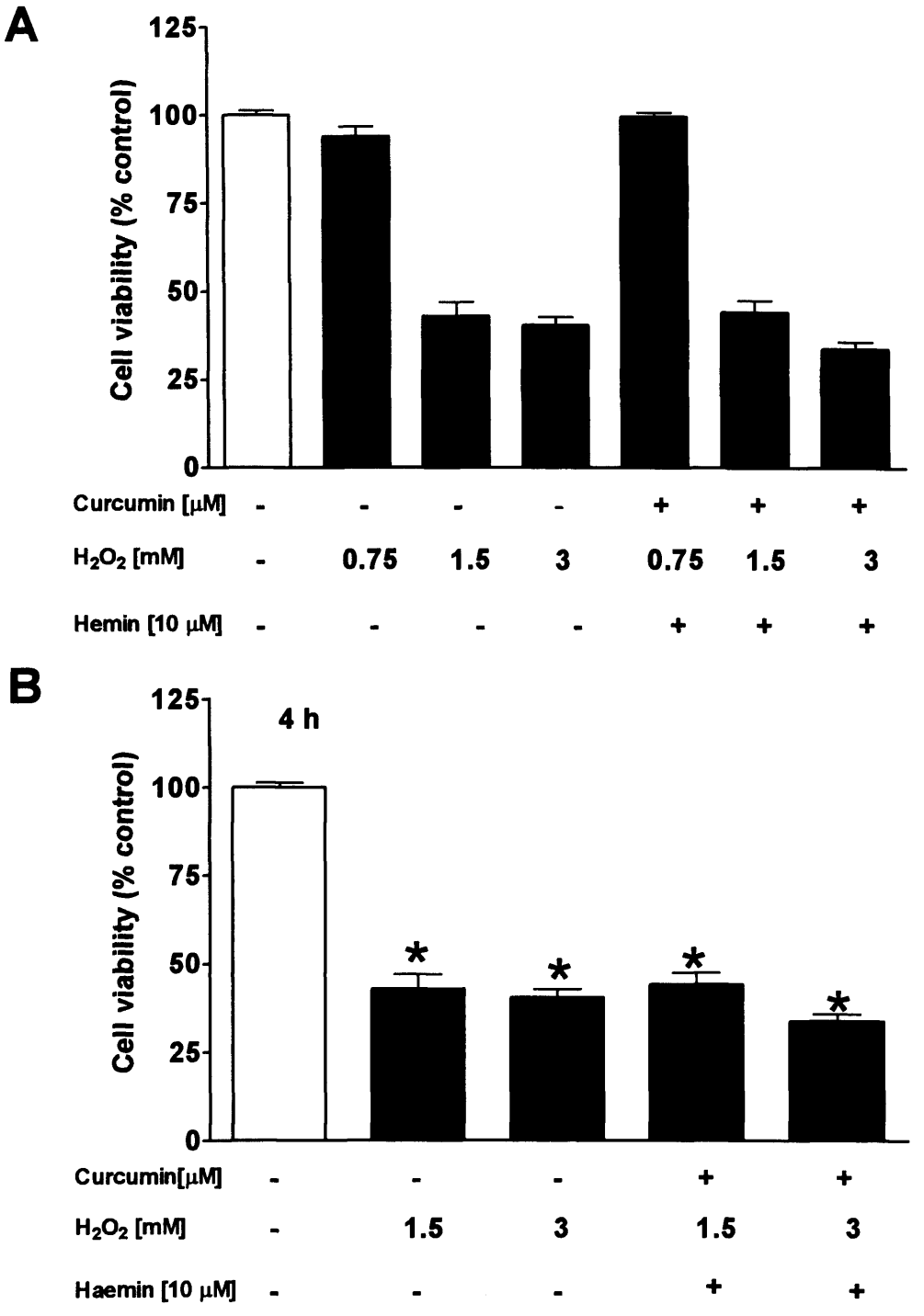



**Figure 6.7: Effect of curcumin and haemin (10 μM) on the $H_2O_2$–mediated oxidative stress**

Girardi cells were pre-treated with of curcumin (15 μM) and haemin (10 μM) for 6 h the medium was then removed and cells were exposed to different concentrations of $H_2O_2$ (1.5 and 3 mM) for 2 h (A), 4 h (B). Cell viability was then determined using Alamar Blue assay. Bars represent the mean ± S.E.M. of 5-6 independent experiments. * $P < 0.001$ vs. control

### 6.5.8 Effect of $H_2O_2$ on LDH Release in Girardi Cells

Treatment of cells with increasing concentrations of $H_2O_2$ (0.75, 1.5, and 3 mM) for 2 (Figure 6.8 A) and 4 h (Figure 6.8 B) did not cause any detectable cytotoxicity, with the percentage of LDH release similar to the level in the control group (3-5%). Therefore, we tested if higher concentrations of $H_2O_2$ would cause ant detectable damage, as shown in (Figure 6.9), treatment with increasing concentrations of $H_2O_2$ (3, 6 or 9 mM) for 2 h did not cause any detectable cytotoxicity measured by LDH release. However, exposure of cells to $H_2O_2$ for 4 h resulted in a concentration-dependent increase in LDH release (7.5, 7.8 and 11 % respectively), (Figure 6.9 B). Treatment of cells with higher concentrations of $H_2O_2$ (3, 6 and 9 mM) for longer time points, i.e. 6 and 8 h, resulted in a time and concentration-dependent increase in LDH release, i.e. 12, 18 and 20% (Figure 6.10 A), and 35, 45, and 55% (Figure 6.10 B) following 6 and 8 h exposure, respectively.

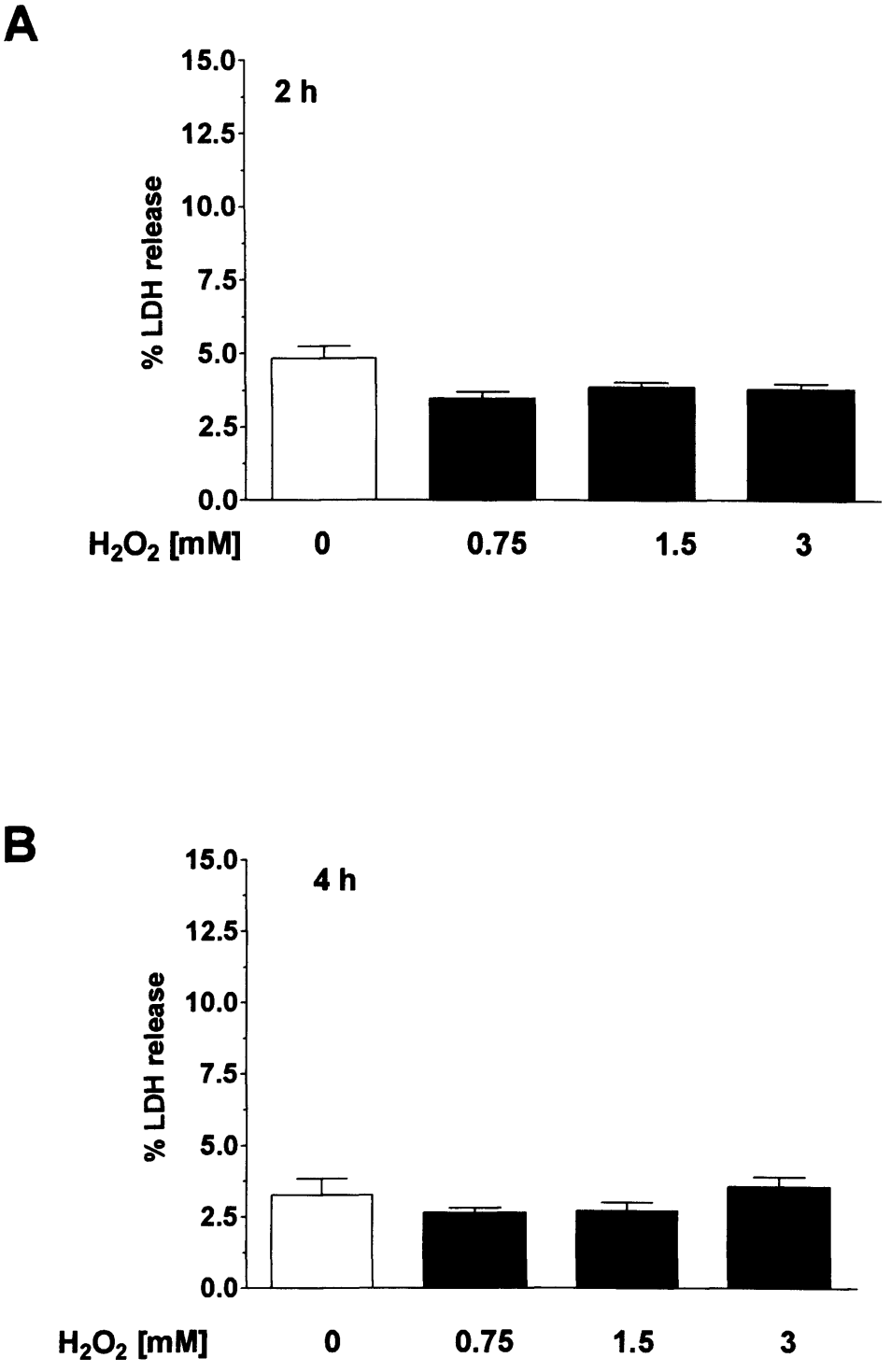



**Figure 6.8: Effect of $H_2O_2$ on the LDH release in Girardi cells**

Girardi cells were exposed to different concentrations of $H_2O_2$ (0.75, 1.5 and 3 mM) for 2 h (A) and 4 h (B). Cell viability was then determined using LDH release. Bars represent the mean ± S.E.M. of 5-6 independent experiments. * $P < 0.001$ vs. control

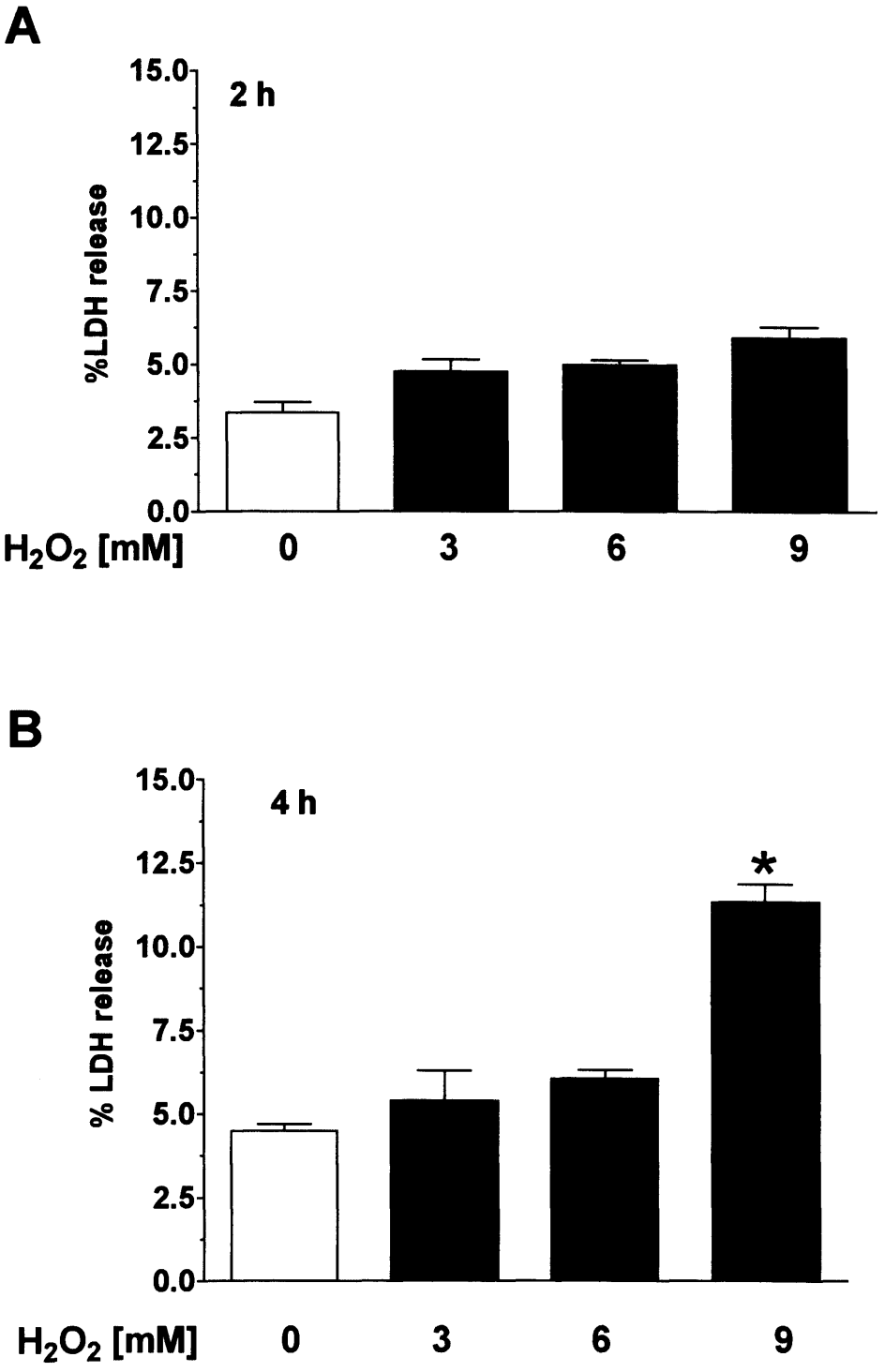



**Figure 6.9: Effect of $H_2O_2$ on the LDH release in Girardi cells**

Girardi cells were exposed to different concentrations of $H_2O_2$ (3, 6 and 9 mM) for 2 h (A) and 4 h (B). Cell viability was then determined using LDH release. Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.05$ vs. control.

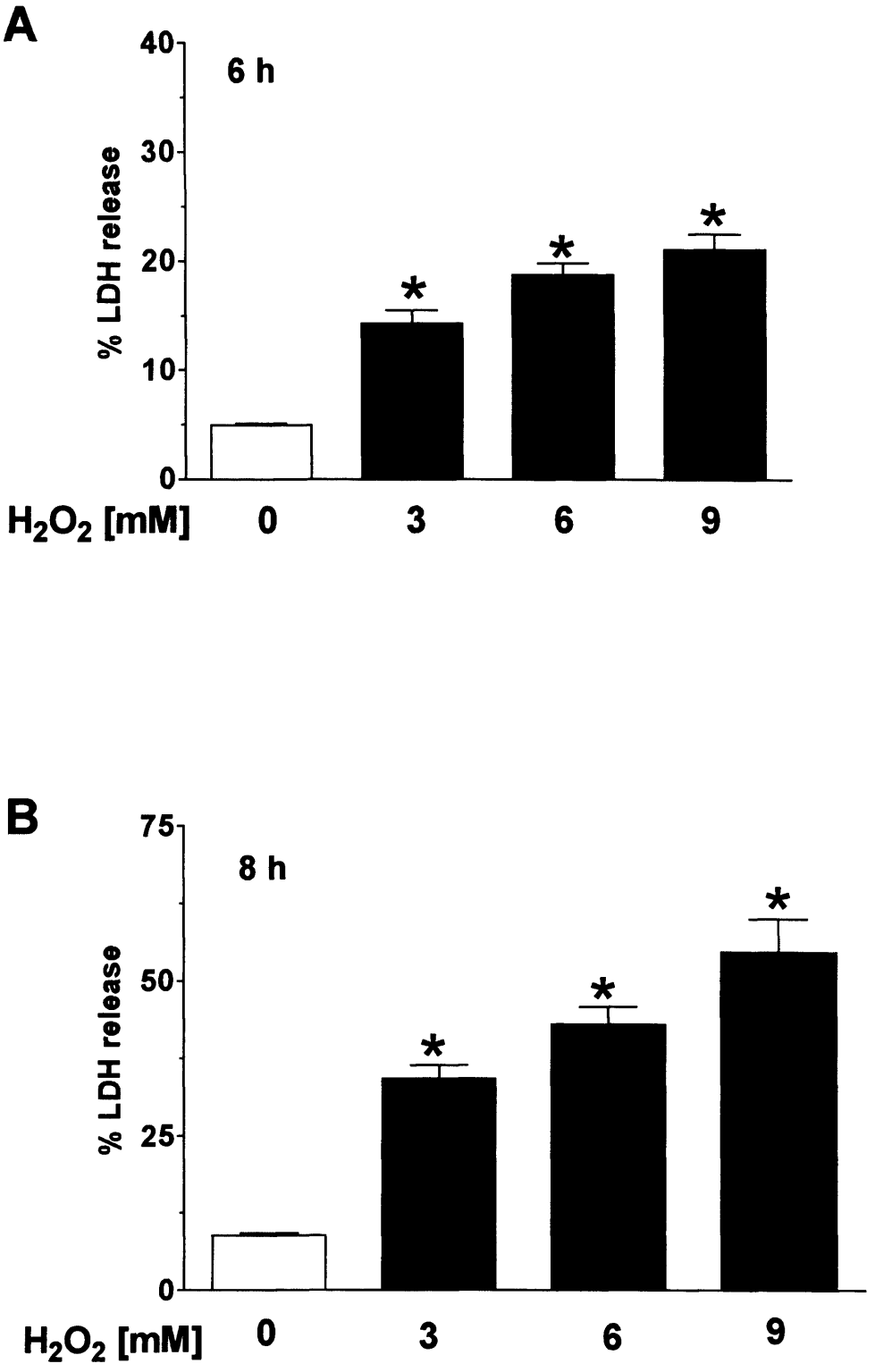



**Figure 6.10: Effect of $H_2O_2$ on the LDH release in Girardi cells**

Girardi cells were exposed to different concentrations of $H_2O_2$ (3, 6 and 9 mM) for 6 h (A) and 8 h (B). Cell viability was then determined using LDH release. Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.001$ vs. control

### 6.5.9 Effect of Curcumin on $H_2O_2$–Mediated Oxidative Stress

Having established that exposure of cells to $H_2O_2$ at 3 and 6 mM for 6 h caused significant cytotoxicity detectable by LDH release; this protocol was employed to study the potential beneficial effects of curcumin on cells exposed to $H_2O_2$ for 6 h. For this purpose, Girardi cells were initially pre-treated with curcumin (15 μM) for 6 h to allow HO-1 induction to take place, cells were then exposed to $H_2O_2$ (3 or 6 mM) for 6 h. Pre-treatment of cells with curcumin (15 μm) significantly attenuated the $H_2O_2$-mediated cytotoxicity evident by a significant improvement in cellular metabolism (Figure 6.11 A). Furthermore, exposure of cells to $H_2O_2$ resulted in a concentration-dependent increase in LDH release; 12, 18 and 20% respectively after 6 h exposure (Figure 6.11 B). Furthermore, pre-treatment of cells with curcumin (15 μM) resulted in a significant reduction in cytotoxicity evident by a decrease in LDH release (Figure 6.11 B). This effect was abolished in the presence of SnPPIX, the inhibitor of haem oxygenase enzymatic activity.

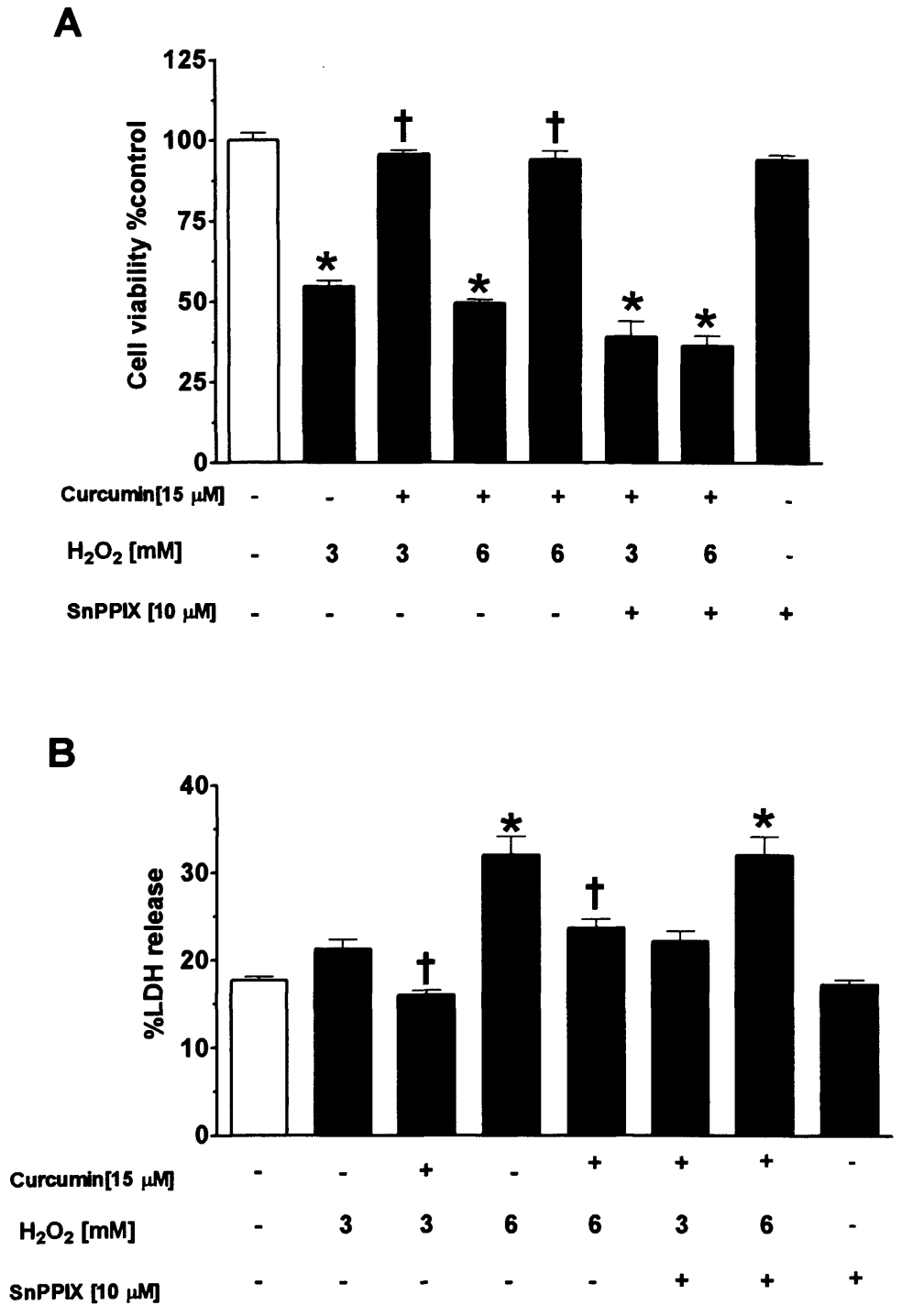



**Figure 6.11: Effect of curcumin on the $H_2O_2$–mediated oxidative stress.**

Girardi cells were pre-treated with of curcumin 15 μM for 6 h and then exposed to different concentrations of $H_2O_2$ (3, and 6 mM) for 6 h in the presence or absence of SnPPIX 10 μM. Cell viability was then determined using Alamar Blue assay (A) and LDH release (B). Bars represent the mean ± S.E.M. of 5-6 independent experiments, * $P < 0.05$ vs. control. † represents $P < 0.05$ vs. $H_2O_2$.

## 6.6 Discussion

ROS from both endogenous and exogenous sources play an important role in the pathogenesis of cardiovascular diseases, such as IHD (Talalay and Talalay, 2001), in which ROS including superoxide, $H_2O_2$ and hydroxyl radicals are found in the myocardium (Dhalla et al., 1999). In the present study, we chose $H_2O_2$ to induce oxidative cell damage in our cultures because it is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals (Mahakunakorn et al., 2003), furthermore, $H_2O_2$ is a major player in the pathogenesis of oxidative stress, since it is generated from nearly all sources of oxidative stress (Chen et al., 2000). Furthermore, exogenous $H_2O_2$ can enter the cells and induce cytotoxicity due to its high membrane permeability (Halliwell et al., 1992). It has been suggested that diets rich in fruits and vegetables are associated with a reduced risk for various cardiovascular diseases, which are mediated by oxidative stress (Morton et al., 2000). These protective effects have been attributed to antioxidant micronutrients such as vitamin C, β-carotene, and vitamin E (Weisburger, 1999). However, scientific attention has also been focused recently on the significance of other minor dietary components, such as natural polyphenolic compounds, as protective agents against diseases (Frusciante et al., 2000). In recent years, several studies emphasized the intrinsic cytoprotective properties of curcumin (Nirmala and Puvanakrishnan, 1996) (Venkatesan, 1998), a polyphenolic phytochemical which possesses antioxidant and free radical-scavenging characteristics (Bonte et al., 1997). Curcumin neutralized ROS including superoxide and hydroxyl radical (Sreejayan and Rao, 1996), it inhibited lipid peroxidation and protected against $H_2O_2$-induced cytotoxicity in renal epithelial cells (Cohly et al., 1998).

Curcumin was found to inhibit lipid peroxidation and lysis in mouse red blood cells challenged with $H_2O_2$ (Toda et al., 1988). Recently, a protective effect of curcumin on $H_2O_2$-induced cell damage in human keratinocytes and human fibroblasts was reported (Phan et al., 2001). In the present study, we evaluated the protective effects of curcumin on $H_2O_2$-induced cell damage in Girardi cells and investigated the possible mechanisms underlying the antioxidant effects of curcumin, with focus on the role of HO-1. Our data demonstrated, for the first time, that curcumin is a potent inducer of haem oxygenase activity and HO-1 protein in Girardi cells, it has been shown before that curcumin is a potent inducer of haem oxygenase activity and HO-1 protein expression in other cell lines such as endothelial cells (Motterlini et al., 2000b) and astrocytes (Scapagnini et al., 2002). Experiments performed to assess cell viability showed no apparent damage after incubation with (5, 15 μM) curcumin for 24 h, however, curcumin at (30 μM) produced a significant increase in cytotoxicity. Our data are in agreement with previous reports showing that curcumin at (1 to 15 μM) does not cause cytotoxicity in vascular smooth muscle cells (Phan et al., 2001). But concentrations of (30–100 μM) appear to mediate pro-oxidant effects and also apoptosis in various cell types (Ahsan et al., 1999). When curcumin at (15 μM) was added simultaneously with various concentrations (3, 6 mM) of $H_2O_2$, it significantly protected Girardi cells against $H_2O_2$-induced cell damage compared to treatment with $H_2O_2$ alone. This is in line with data in the literature which demonstrated that curcumin protected cells against $H_2O_2$-induced cell damage (Cohly et al., 1998). Incubation of cells for 6 h with curcumin resulted in enhanced resistance to oxidative damage; this effect was attributable to induction of HO-1 as the inhibitor of haem oxygenase activity

(SnPPIX) markedly reduced cytoprotection by curcumin treatment. The antioxidant properties of this phytochemical are in essence related to its ability to stimulate a defensive intracellular enzymatic pathway, namely HO-1(Moskaug et al., 2005). In addition, it is possible that the protective effect of curcumin against the $H_2O_2$-induced cytotoxicity may partly result from its intrinsic antioxidant and free radical scavenging properties; since pre-treatment with curcumin (removal of the medium which contains curcumin) was unable to protect Girardi cells from $H_2O_2$-induced oxidative damage. The addition of haemin to the experimental protocol was aimed at investigating any possible synergism between curcumin and haemin to protect cardiac cells against cytotoxicity induced by $H_2O_2$. In contrast, pre-treatment with curcumin and haemin (which is known pro-oxidant *per se*) rendered the cells more susceptible to cytotoxicity. It is possible that both curcumin and haemin are taken up by cells and then elicit changes that increase the susceptibility of cells to oxidative stress induced by $H_2O_2$. The dual actions of curcumin suggest that pretreatment with curcumin sensitizes them to oxidative damage induced by $H_2O_2$. Curcumin shows antioxidant and free radical scavenging activities, which are usually considered to protect cells from oxidative stress (Bonte et al., 1997). However, this polyphenolic compound has been reported to induce significant DNA damage by free radical generation (Ahsan et al., 1999). $H_2O_2$ is believed to cause cytotoxicity by reacting with the cell membrane and producing lipid peroxidation (Salahudeen, 1995), since cellular membranes are the richest source of lipids; plasma membranes are important targets for cell injury in $H_2O_2$ (Salahudeen et al., 2000). Therefore LDH release assay was chosen in this study to assess cytotoxicity, because LDH release usually correlates to cellular

membrane damage (Venkatesan, 1998), and therefore; LDH release is suggested to be a reliable method of assessing cytotoxicity (Bartels-Stringer et al., 2003). However, while $H_2O_2$ at low concentrations (1.5 and 3 mM) for 2, 4 and 6 h incubation caused significant decrease in the metabolism in cells (assessed by Alamar Blue) (40 and 35 % compared to control group Figure 6.4), $H_2O_2$ at these concentrations (1.5 and 3 mM) did not result in any LDH release in the cells. Therefore higher concentrations of $H_2O_2$ (6 and 9 mM) incubated for longer incubation times (6 and 8 h) (Figure 6.10) were needed to induce detectable cytotoxicity using the LDH release. It is tempting to speculate that in our experimental approach, Alamar Blue is a more sensitive assay in the detection of cellular derangements induced by $H_2O_2$ than LDH release. However; cells exposed to $H_2O_2$ are more subjected to damage to the cell membrane, rendering the membrane more permeable to LDH, which is reflected by an increase in the LDH release by cells. So it is tempting to speculate that LDH is a reliable assay for determination of $H_2O_2$-mediated cytotoxicity when higher concentrations of $H_2O_2$ are used, i.e. (3-6 mM).

In this study, we suggest that the use of curcumin as a therapeutic agent to mitigate cardiovascular disease and other vascular dysfunction-mediated by oxidative stress. This cytoprotective action is related to the ability of curcumin to act as a scavenger of ROS, and to a lesser extent, up-regulate HO-1 expression.

# 7 CURCUMIN MODULATES THE EXPRESSION OF HO-1 UNDER HYPOTHERMIC CONDITIONS

## 7.1 Introduction

Heart transplantation is the treatment of choice for end stage cardiac disease (Jahania et al., 1999). Currently, a greater availability of harvested human hearts is precluded by the short period of cold ischaemia tolerated by this organ (Jahania et al., 1999). To make organs tolerant to hypothermia, blood is removed and replaced with an appropriate hypothermic preservation solution (Muhlbacher et al., 1999). The strategy commonly used to reduce ischaemic injury during cold storage is the rapid flushing, and cooling of the organs to 4°C using preservation solutions (Southard and Belzer, 1995). The methods used to preserve organs are based on suppression of metabolism by hypothermia; cold storage slows the cellular metabolism and extends cellular viability (Muhlbacher et al., 1999). However, prolonged storage is still associated with cold-induced cell and tissue injury (Kuznetsov et al., 2004). Several mechanisms mediate cold storage–associated injury, for example, reduced ATP levels which lead to cell swelling, and anaerobic metabolism, resulting in intracellular lactic acidosis (McLaren and Friend, 2003) (Kuznetsov et al., 2004). For both cold storage and reperfusion, ROS play an important pathogenetic role, mainly by provoking lipid peroxidation by incorporating into membrane lipid bilayers (Bartels-Stringer et al., 2003). Preservation solutions have been designed to slow down the rate at which reactions leading to I/R injury take place (Southard and Belzer, 1995). For the latter reason, most preservation solutions are high in potassium (to prevent loss of cellular K in the absence of Na, K-ATPase activity) and include a

colloid to prevent cell swelling (Michel et al., 2002). However, these solutions are only poorly protective against I/R injury. This is entirely explicable, if one considers all the complex mechanisms of cold-induced injury. The antioxidants present in some of the solutions, such as glutathione, are not directed against the mechanism involved and cannot be expected to be present where the antioxidant would be required (Muhlbacher et al., 1999). Glutathione (oxidized after storage of the solution) remains largely extracellular and is thus not able to protect against the species generated intracellularly (Salahudeen et al., 2000). Similarly, mannitol, which is known to be a hydroxyl radical scavenger, cannot protect against intracellularly generated hydroxyl radicals because of its lack of membrane permeability, and because hydroxyl radical scavenging requires excessively high concentrations (>100mM) (Muhlbacher et al., 1999). Therefore, the composition of the preservative appears to be a critical determinant of the tolerance of the organ to hypothermic storage (Salahudeen et al., 2000). Thus, it is of importance to add agents which protect the organs from the damage which occurs during cold storage. Celsior solution, a new preservation solution was introduced in heart transplantation (Michel et al., 2002). Preliminary data for Celsior solution in the preservation of thoracic organs showed an improved graft function after ischaemia, and reperfusion in comparison to other preservation solutions (Michel et al., 2002) (Jahania et al., 1999). Treatment with the polyphenolic compounds was associated attenuation of renal inflammation in a rat model of transplantation (Jones and Shoskes, 2000). In endothelial cells Fuller *et al.* showed that addition of curcumin abrogated expression of adhesion molecules (E-selectin and ICAM- 1 (intercellular adhesion molecule 1) (Fuller et al., 2003). Renal epithelial cells

pre-treated with curcumin were more resistant to oxidative damage during a programmed change in temperature under hypoxia (Balogun et al., 2003a). Based on the hypothesis that curcumin would provide a defense mechanism against cardiac I/R injury, this study examined the efficacy of curcumin in protection of cardiac cells against prolonged cold preservation.

## 7.2 Objectives

To determine whether curcumin, a plant-derived polyphenolic compound and a potent inducer of HO-1 could up-regulate haem oxygenase activity and HO-1 mRNA expression under hypothermic conditions, and to investigate whether the addition of curcumin to a commercial preservation solution (Celsior) improved and extended cardiac preservation.

## 7.3 Material and Methods

### 7.3.1 Reagents

Haemin (ferriprotoporphyrin IX chloride) and tin protoporphyrin (SnPPIX) were obtained from Porphyrin Products INC (Logan, Utah, USA). Curcumin and all other reagents were purchased from Sigma. Alamar Blue reagent was obtained from Serotec (Oxford, UK). Polyclonal antibodies were purchased from Bio-Quote Ltd. PCR primers were purchased from Amersham. Celsior solution (Table 7-1) was obtained from IMTIX SANGSTAT, Lyon, France.

**Table 7-1: Composition of Celsior solution**

| Composition | Weight (g) | Concentration (mM) |
|---|---|---|
| **Mannitol** | 10.930 | 60 |
| **Lactobionic acid** | 28.664 | 80 |
| **Glutamic acid** | 2.942 | 20 |
| **Sodium hydroxide** | 4.00 | 100 |
| **Calcium chloride** | 0.037 | 0.25 |
| **Potassium chloride** | 1.118 | 15 |
| **Magnesium chloride** | 2.642 | 13 |
| **Histidine** | 4.650 | 30 |
| **Glutathione** | 0.921 | 3 |

### 7.3.2 Cell Culture

Girardi cells (human cardiac myoblasts) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK) and cultured in Dulbecco's modified Eagle's medium containing, 3.5 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and supplemented with 10% fetal bovine serum and 1% non-essential amino acids. Cells were grown in 75-$cm^2$ flasks and maintained at 37°C in a humidified atmosphere of air and 5% $CO_2$. Confluent cells were exposed to various concentrations of curcumin and haemin, and haem oxygenase activity and HO-1 protein expression and mRNA were determined at different times after treatment. Cells were also incubated with different concentrations of curcumin in conditions of programmed changes in temperature using a cooled incubator (Sanyo, Model MIR-153), where a temperature controller allowed setting and programming the temperature of the interior of the cabinet. Within the incubator, cells were placed in an air-tight Plexiglas chamber (Billups-Rothenberg, Del Mar, CA) and flushed with a mixture of 21% $O_2$/5% $CO_2$/74% $N_2$ to simulate "normoxic" incubation.

### 7.3.3 Experimental Protocol

The cells were studied upon confluence, in 24-well plates. The groups included parallel control cells kept in Dulbecco's modified Eagle's medium (DMEM), and cells subjected to cold storage in CS solution over 4, 6, 8 and 12 h. Cytotoxicity was determined using LDH release, Trypan Blue exclusion was used to determine cell membrane integrity and Alamar Blue to determine the effect of cold storage on cellular metabolism. Before cold storage, the prevailing cell culture medium was replaced by Celsior solution; cells were then placed in a temperature monitored refrigerator at 4°C. To determine the effect of rewarming

on cold storage-induced cell injury, cells cold-stored for 6 h were subjected to 0, 1 and 2 h of rewarming in the incubator at 37°C before cytotoxicity was assessed using LDH release, Trypan Blue and Alamar Blue. To determine the effect of curcumin on the cold–induced cellular injury, similar sets of experiments were performed, in which cells were exposed to cold storage in Celsior solution over 6 h in the presence of curcumin 15 μM. Furthermore, cells exposed to 6 h cold storage were then rewarmed for 1 h. To investigate the involvement of haem oxygenase, similar sets of experiments were conducted in the presence of SnPPIX (10 μM), an inhibitor of haem oxygenase activity. In a different set of experiments, cells were incubated with curcumin (5-30 μM) under a programmed change in temperature which included exposure for 3 hours at 37°C followed by 3 hours at 4°C in Celsior solution for an additional 3 h. After these treatments, cells were harvested for the determination of haem oxygenase activity and HO-1 mRNA expression. Additional experiments were performed to simulate the oxidative stress which concurs during the rewarming. For this purpose, cells exposed to the programmed change in temperature were further incubated with paraquat, a known generator of free radicals (1, 1.5 mM) for 18 h; cellular damage was assessed by using the Alamar Blue assay. To address the contribution of HO-1, a parallel set of experiments was performed using siRNA for HO-1.

### 7.3.4 Cell Viability/Alamar Blue Assay

Cell viability was determined using an Alamar Blue assay kit, it was carried out as previously described in Section 2.3.1.

### 7.3.5 LDH Assay

Extracellular, i.e., released, LDH activity was measured using cytotoxicity detection kit (Roche) as previously described in section 2.3.2.

### 7.3.6 Trypan Blue Assay

Trypan Blue exclusion was performed as previously described in section 2.3.3.

### 7.3.7 Haem Oxygenase Activity Assay

Haem oxygenase activity was determined at the end of each treatment as described previously in Section 2.4.3.

### 7.3.8 RNA Isolation

RNA isolation was performed using Purescript® RNA Isolation Kit (Gentra, Minneapolis, MN 55441 USA). RNA isolation was performed according to the manufacturer instruction, as previously described in (section 2.5.6).

### 7.3.9 Measurement of HO-1 mRNA by RT-PCR

PCR was performed as previously described in (section 2.5.7).

### 7.3.10 Transfection of Girardi cells with HO-1 siRNA

Girardi cells were grown in twelve-well plates and transiently transfected with HO-1 siRNA mixed with the appropriate transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions and as previously described in Section 2.5.4. After incubation at 37°C for 30 h, cells were exposed to hypothermia for 4, 6, 8, 12, and 24 h in the presence or absence of curcumin (15 μM) as described in the Experimental Protocol. Samples were then collected and analyzed for LDH release.

## 7.4 Statistical Analysis

Differences among the groups were analyzed using one-way analysis of variance combined with the Bonferroni test. Values were expressed as mean ± S.E.M., and differences between groups were considered to be significant at $p < 0.05$.

## 7.5 Results

### 7.5.1 Time Course for Cold Storage-Induced Cytotoxicity

Exposure of Girardi cells at 4°C in Celsior solution resulted in a time-dependent increase in LDH release compared with warm control cells, i.e. cells kept in cell culture medium at 37°C. The increase of LDH release in hypothermia was strikingly higher than the spontaneous release found in cells kept in cell culture medium at 37°C. For example, the LDH release in cells after 6 h of cold storage was over 65%, whereas in a similar time period the LDH release at 37°C was close to 5% (Figure 7.1 A). This was accompanied by an increase in the uptake of Trypan Blue dye by the cells. As can be seen in (Figure 7.1 B) there was a time-dependent increase in the uptake of the dye, reaching the maximum uptake, i.e. cytotoxicity (100%) after 8 and 12 h incubation at 4°C. Furthermore, there was a time-dependent decrease in cellular metabolism, 50 %, 45%, and 20 % after 4, 6 and 8 h cold storage respectively. Interestingly, cells exposed to 12 h cold storage did not exhibit any detectable metabolic activity by the Alamar Blue Assay (Figure 7.1 C).

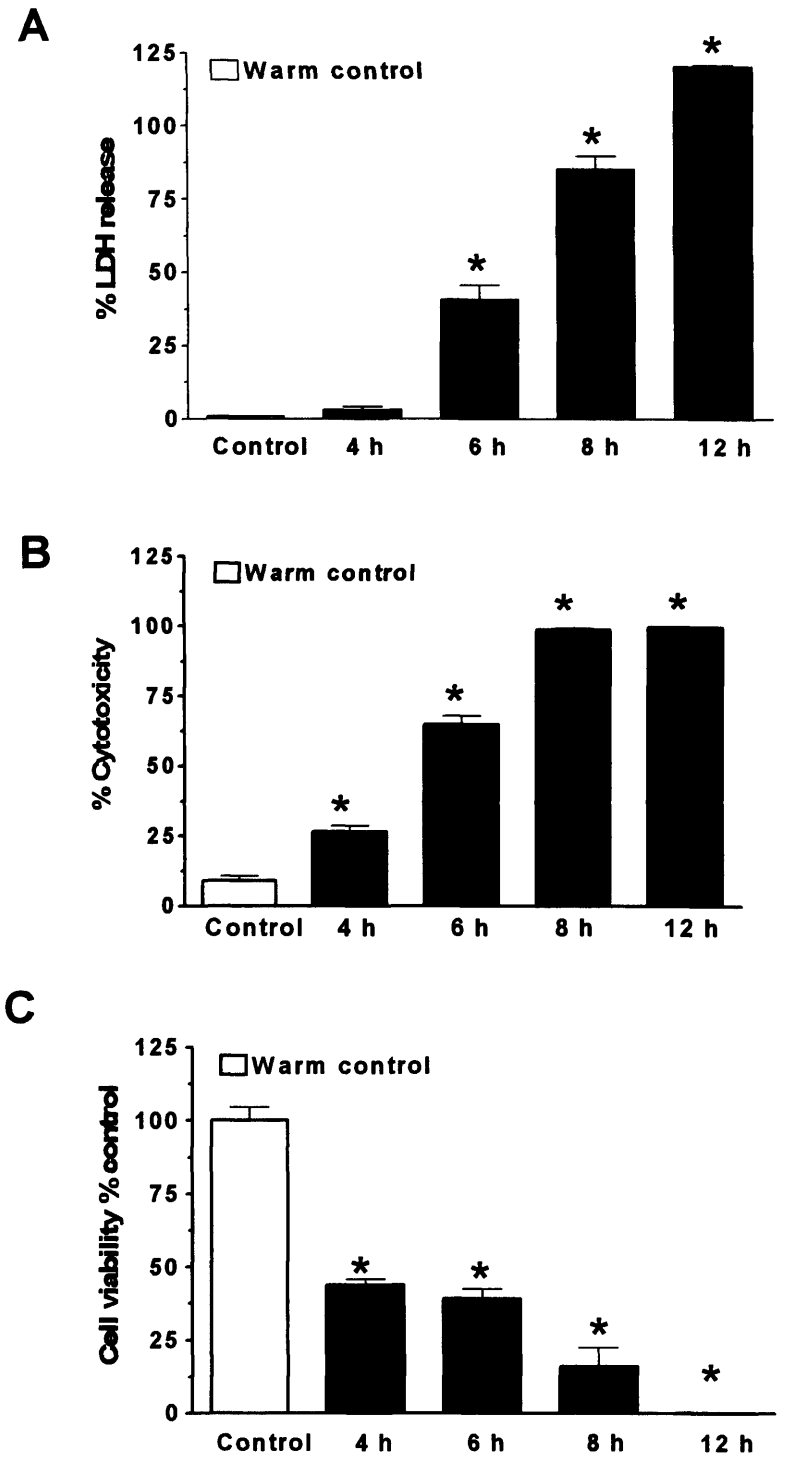



**Figure 7.1: Time course for the cold storage-induced cytotoxicity**

Girardi cells were exposed to 4°C for 4, 6, 8 and 12 h in Celsior solution. Cytotoxicity was then assessed using LDH release (A) Trypan Blue exclusion (B) and Alamar Blue (C). The control group (warm control) is represented by cells kept in cell culture medium at 37°C. Each bar represents mean ± S.E.M. of 6 independent experiments. * $P < 0.001$ vs. warm control.

### 7.5.2 Cytotoxicity in Girardi Cells Exposed to 6 h Cold Storage and then Rewarming

Having established that cold storage after 6 h results in significant cytotoxicity without reaching irreversible (100% cytotoxicity), this time point was chosen to study the effect of rewarming on cellular damage. Rewarming for 1 or 2 h, i.e., replacement of Celsior solution after a 6 h cold storage with a regular cell culture medium and re-incubating the cells at 37°C. Exposure of cells to this protocol caused additional marked increase in LDH release (Figure7.2 A), increased cytotoxicity detected by Trypan Blue (Figure7.2 B) and decreased metabolism as assessed by Alamar Blue (Figure7.2 C). Cellular damage was further aggravated during rewarming, reaching maximal levels as detected by Trypan Blue uptake after 2 h rewarming (Figure7.2 C).

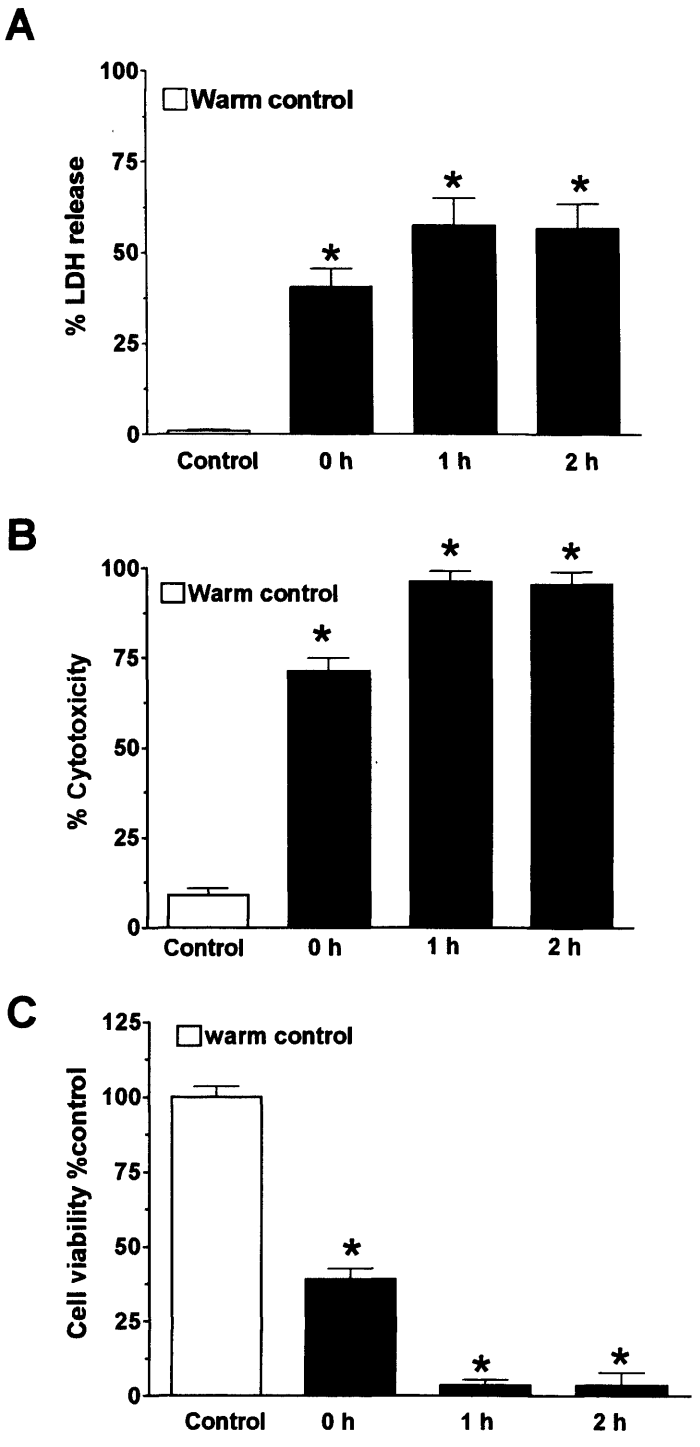



**Figure7.2: Time course for rewarming-induced cytotoxicity**

Girardi cells were exposed to 4 °C for 6 h in Celsior solution followed by rewarming (replacement of Celsior solution after a 6-h cold storage with cell culture medium and re-incubating the cells at 37°C). Cytotoxicity was then assessed using LDH release (A) Trypan Blue exclusion (B) and Alamar Blue (C). The control group is represented by cells kept in cell culture medium at 37°C. Each bar represents mean ± S.E.M. of 6 independent experiments. * $P < 0.001$ vs. warm control.

### 7.5.3 The Effect of Curcumin on Hypothermia and Rewarming-Induced Cytotoxicity in Girardi Cells

Curcumin at (15 μM) concentration completely suppressed the 6-h cold-induced LDH release (Figure 7.3 A), decreased Trypan blue uptake by the cells (Figure 7.3 B) and normalized the cellular metabolism (Figure 7.3 C). When SnPPIX was added, the protective effects of curcumin on LDH release were not diminished (Figure 7.3 A). However, there was a mild attenuation of this effect as observed by Trypan Blue exclusion (Figure 7.3 B) and cellular metabolism (Figure 7.3 C). Similarly, curcumin completely reversed the 1 h rewarming-induced increase in LDH release (Figure 7.4 A), decreased Trypan blue uptake by Girardi cells (Figure 7.4 B) and normalized the cellular metabolism (Figure 7.4 C). The protective effects of curcumin on LDH release was not diminished in the presence of SnPPIX, however, there was a mild attenuation of this effect as observed by Trypan blue exclusion (Figure 7.4 B) and cellular metabolism (Figure 7.4 C).

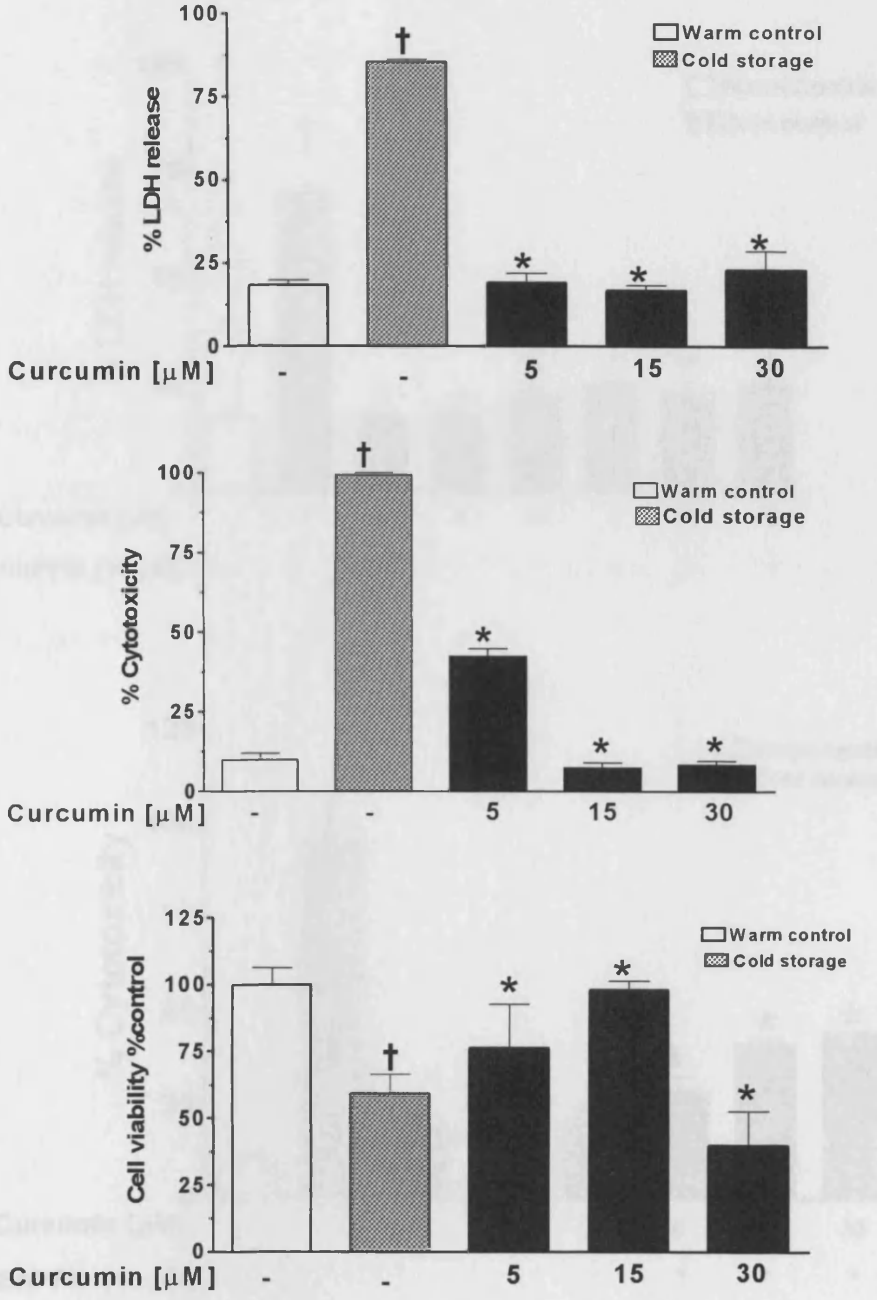



**Figure 7.3: The effect of curcumin on the cold storage cytotoxicity in Girardi cells**

Girardi cells were exposed to 4°C for 6 h in Celsior solution in the presence or absence of curcumin (15 μm). Cytotoxicity was then assessed using LDH release (A) Trypan Blue exclusion (B) and Alamar Blue (C). The control group is represented by cells kept in cell culture medium at 37°C. Each bar represents mean ± S.E.M. of 6 independent experiments. † represents $P < 0.001$ vs. warm control, * $P < 0.001$ vs. cold control.

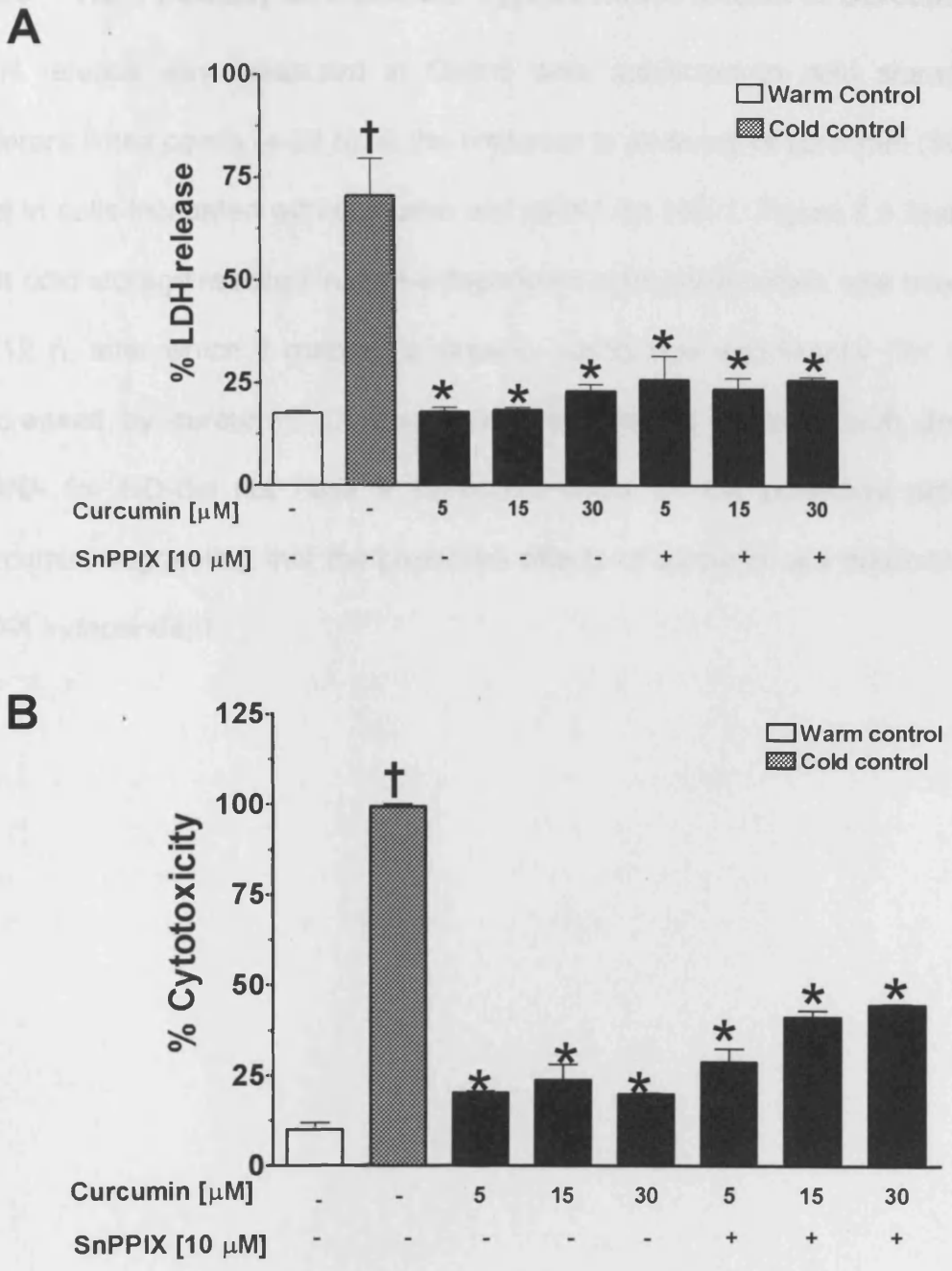



**Figure 7.4: The effect of curcumin on the cold storage cytotoxicity in Girardi cells**

Girardi cells were exposed to 4oC for 6 h in Celsior solution, followed by rewarming at 37°C for 1 h in the presence or absence of curcumin 15 μm and SnPPIX (10 μM). Cytotoxicity was then assessed using LDH release Trypan Blue exclusion (B). The control group is represented by cells kept in cell culture medium at 37°C. Each bar represents mean ± S.E.M. of 6 independent experiments. † represents $P < 0.001$ vs. warm control, * $P < 0.001$ vs. cold control.

### 7.5.4 HO-1 Partially Mediates the Cytoprotective Effects of Curcumin

LDH release was measured in Girardi cells subjected to cold storage for different times points (4-24 h), in the presence or absence of curcumin (15 μM), and in cells incubated with curcumin and siRNA for HO-1. Figure 7.5 illustrates that cold storage resulted in a time-dependent cytotoxicity which was maximum at 12 h, after which it reached a plateau, which was significantly ($P < 0.001$) decreased by curcumin. Consistent with the results obtained with SnPPIX, siRNA for HO-did not have a significant effect on the protective action of curcumin, suggesting that the protective effects of curcumin are predominantly HO-1 independent.

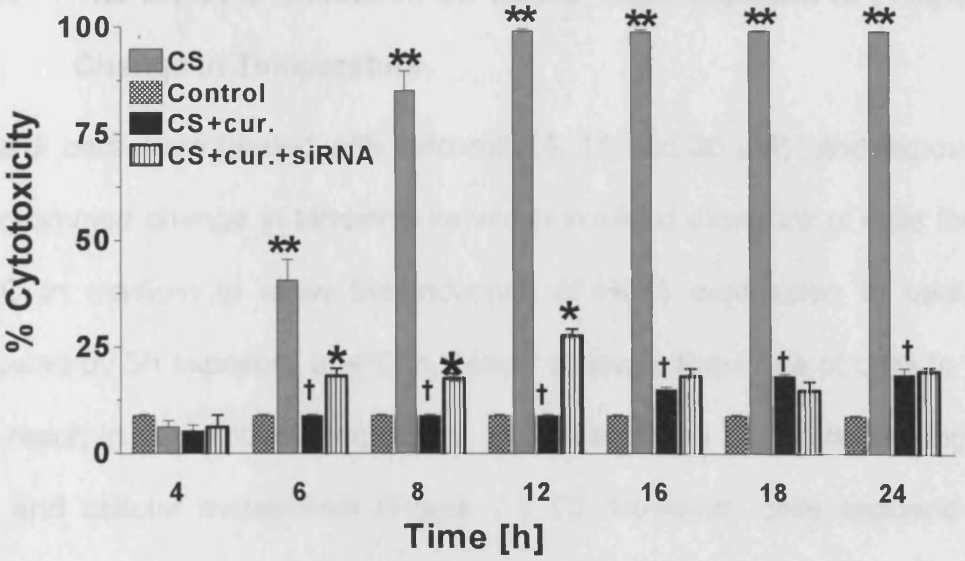



**Figure 7.5: HO-1 mediates the protective effects of curcumin**

Girardi cells were transfected with HO-1 siRNA; (CS): represents cells exposed to 4°C h (4-24 h) in Celsior solution, cells exposed to this protocol in the presence of curcumin 15 µM is represented by (cur.). Cytotoxicity was then assessed using LDH release. The warm control (Control) is represented by cells kept in cell culture medium at 37°C. Each bar represents mean ± S.E.M. of 6 independent experiments. ** $P < 0.001$ vs. warm control, † $P < 0.001$ vs. cold control. * $P < 0.05$ vs. curcumin.

### 7.5.5 The Effect of Curcumin on Girardi Cells Exposed to Programmed Change in Temperature

Girardi cells were treated with curcumin (5, 15 and 30 μM) and exposed to a programmed change in temperature which involved exposure of cells for 3 h at 37°C in medium to allow the induction of HO-1 expression to take place, followed by 3h exposure at 4°C in Celsior solution. Exposure of cells to this did not result in any evident cytotoxicity, as measured by LDH release (Figure 7.6 A), and cellular metabolism (Figure 7.6 C). However, cells exposed to this programmed change in temperature showed signs of toxicity evident by a significant increase (30% compared to warm control) in the uptake of the Trypan Blue dye (Figure 7.6 B). Preconditioning of cells with increasing concentrations of curcumin significantly reduced the cold storage-induced cellular membrane derangement detected by Trypan Blue uptake (Figure 7.6 B).

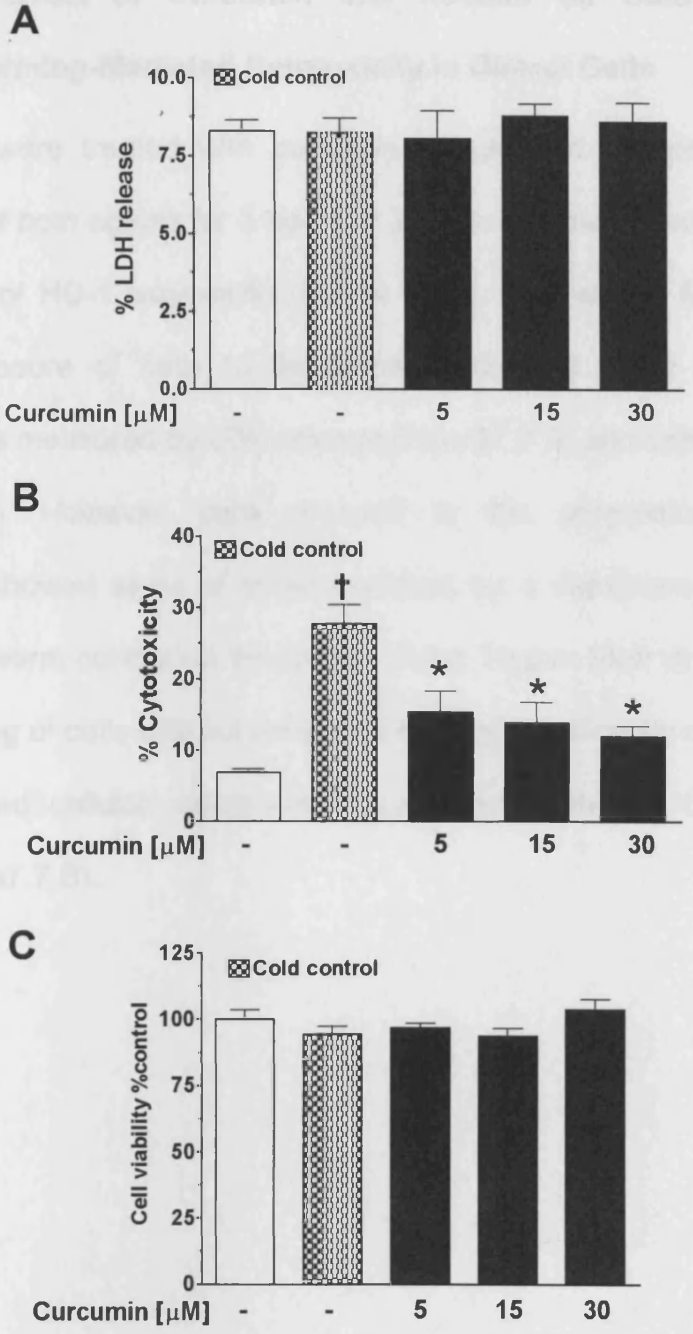



**Figure 7.6: The effect of curcumin on cell viability in Girardi cells exposed to a programmed change in temperature**

Girardi cells were exposed to various concentrations of curcumin (5, 15 or 30 μM), initially for 3 h at 37°C, followed by 3 h-incubation at 4°C. LDH release (A) Trypan Blue (B) and Alamar Blue assays (C) were performed. The control group is represented by cells incubated with medium alone (0 μM). Each bar represents mean ± S.E.M. of 6 independent experiments. * $P < 0.05$ vs. cold control. † $P < 0.001$ vs. warm control.

### 7.5.6 The Effect of Curcumin and Haemin on Cold Storage and Rewarming-Mediated Cytotoxicity in Girardi Cells

Girardi cells were treated with curcumin 15 μM and haemin 5 μM or the combination of both agents for 3 hours at 37°C in complete medium to allow for the induction of HO-1 expression to take place, then at 4°C for 3h in Celsior solution. Exposure of cells to this protocol did not result in any evident cytotoxicity, as measured by LDH release (Figure7.7 A) and cellular metabolism (Figure7.7 C). However, cells exposed to this programmed change in temperature showed signs of toxicity evident by a significant increase (30% compared to warm control) in the uptake of the Trypan Blue dye (Figure7.7 B). Preconditioning of cells with curcumin and haemin significantly reduced the cold storage-induced cellular membrane derangement detected by Trypan Blue uptake (Figure7.7 B).

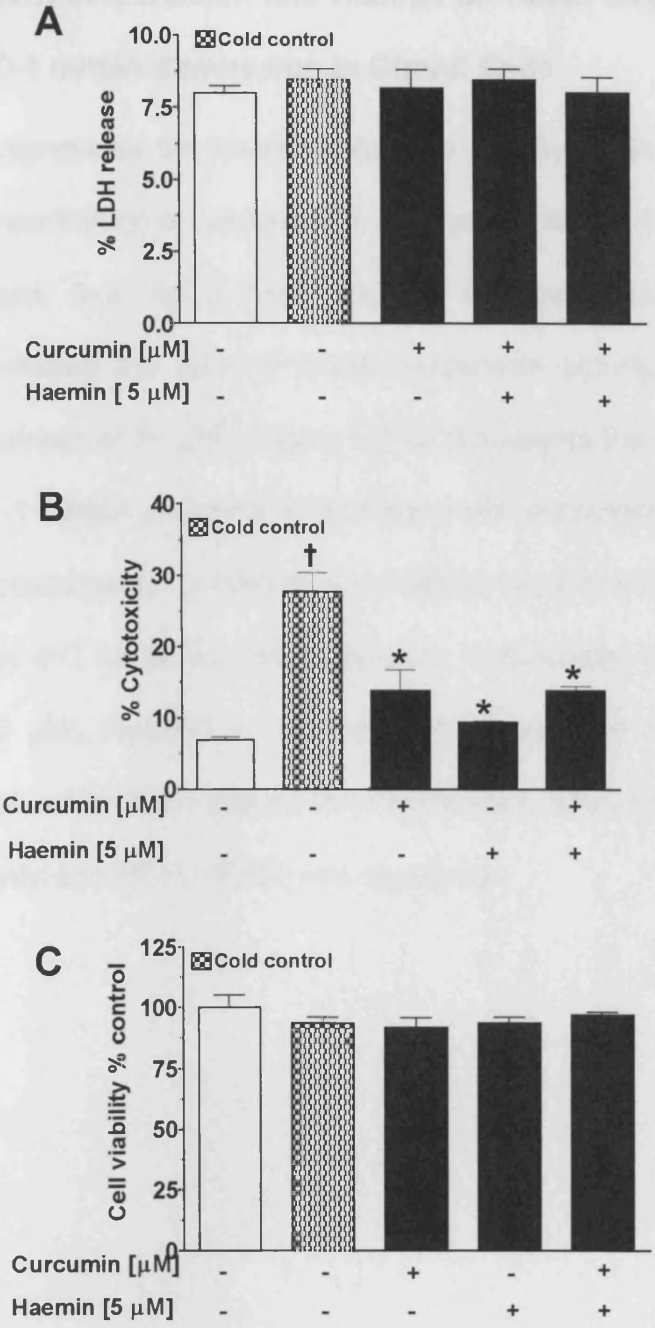



**Figure7.7 The effect of curcumin and haemin on cell viability in Girardi cells exposed to a programmed change in temperature**

Girardi cells were exposed to curcumin 15 μM in the presence or absence of haemin 5 μM (B), initially for 3 hours at 37oC, then 3 hours at 4oC. LDH release (A) Trypan blue (B) and Alamar Blue (C) assays were performed. The control group is represented by cells incubated with medium alone (0 μM). Each bar represents mean ± S.E.M. of 6 independent experiments. * $P < 0.001$ vs. cold control. † $P < 0.001$ vs. warm control.

### 7.5.7 The Effect of Curcumin and Haemin on Haem Oxygenase Activity and HO-1 mRNA Expression in Girardi Cells

(Figure 7.8 A) represents the haem oxygenase activity in Girardi cells treated with various concentration of curcumin (5, 15 and 30 μM) for 3 hours at 37°C in complete medium, then for 3 hours at 4°C in Celsior solution. Curcumin significantly increased the level of haem oxygenase activity with a maximal value being observed at 30 μM. (Figure 7.8 B) represents the haem oxygenase activity and HO-1 mRNA in Girardi cells treated with curcumin (15 μM), haemin (5 μM) or the combination of both agents added for 3 h at 37°C in complete medium, then at 4°C for 3h in Celsior solution. Individually, curcumin (15 μM) and haemin (5 μM) resulted in a significant increase in haem oxygenase activity, however, when both agents were combined, further increase in haem oxygenase activity and HO-1 mRNA was observed.

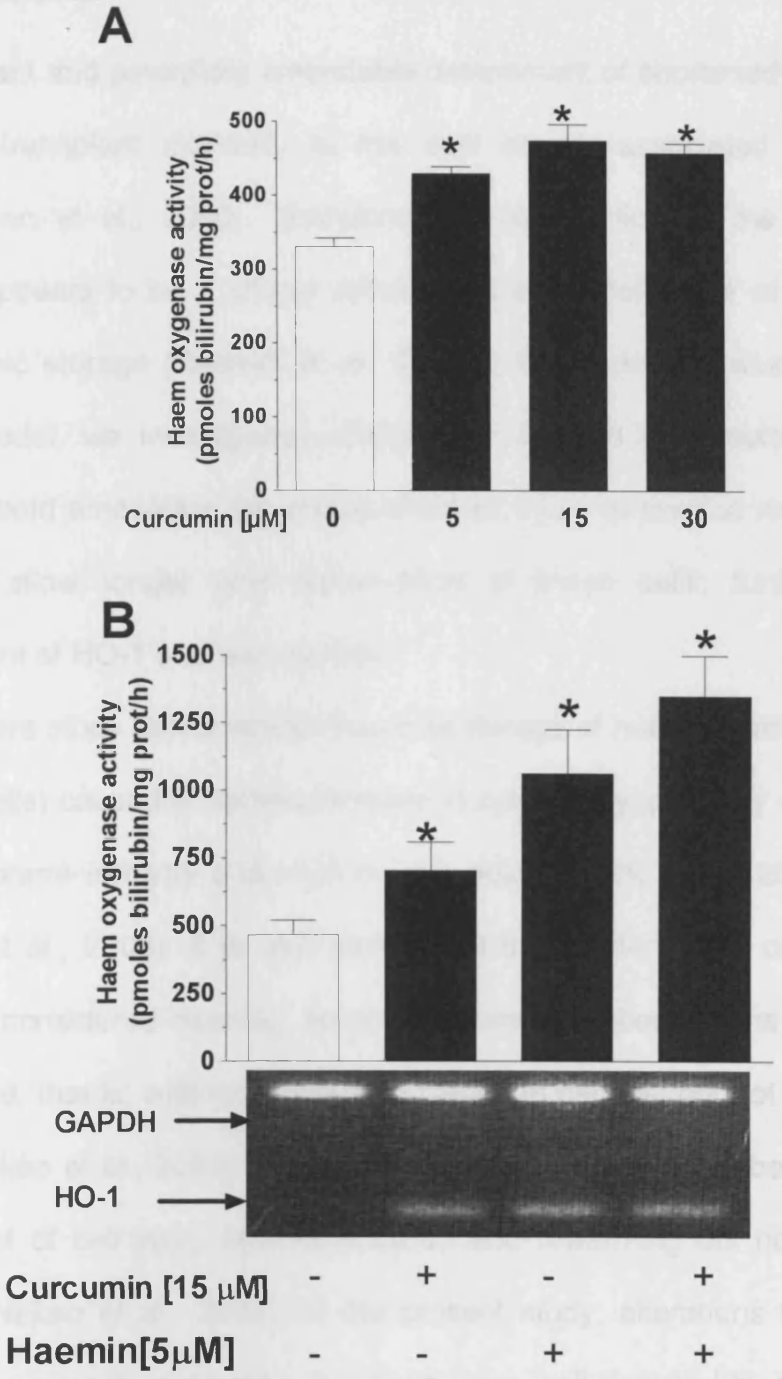



**Figure 7.8: Effect of change in temperature on curcumin-mediated induction of haem oxygenase activity and HO-1 mRNA expression**

Girardi cells were exposed to various concentrations of curcumin (5, 15 and 30 μM) (A) or curcumin 15 μM in the presence or absence of haemin 5 μM (B). Cells were exposed initially for 3 h at 37oC, then 3 h at 4oC. Haem oxygenase activity and HO-1 mRNA expression were determined. Each bar represents mean ± S.E.M. of 6 independent experiments. * $P < 0.001$ vs. Control.

## 7.6 Discussion

An important and potentially amendable determinant of shortened graft survival and post-transplant morbidity is the cold storage-associated organ injury (Salahudeen et al., 2000). Therefore, the composition of the preservation solution appears to be a critical determinant of the tolerance of the organ to hypothermic storage (Redaelli et al., 2002a). In the present study with a cell culture model, we investigated whether the addition of curcumin to Celsior solution could ameliorate the cold-associated injury in cardiac myoblasts, and therefore allow longer cold preservation of these cells; furthermore, the involvement of HO-1 was also studied.

The present study demonstrated that cold storage of human cardiac myoblasts (Girardi cells) caused a marked increase in cytotoxicity, primarily due to loss of cell membrane integrity and necrotic cell death (Clerk et al., 2003) (Bartels-Stringer et al., 2003). It is well established that cold-induced organ injury is generally considered necrotic, recent experimental observations suggest that cold *per se*, that is, without reperfusion results in necrotic but not apoptotic cell death (Nakao et al., 2003). Apoptotic cell death was found to be an important component of cell injury after reperfusion and rewarming but not during cold storage (Nakao et al., 2003). In the present study, alterations to membrane integrity as a result of necrotic cell death were well depicted by the release of intracellular LDH, and increase in the uptake of the Trypan Blue dye. The injury observed at 6 hours may be attributable to the well-described mechanism of cell injury caused by cold storage: mitochondrial ATP synthesis decreases, tissue acidosis occurs, and acidic proteases are activated, which leads to degradation of proteins and undermining of cell membrane integrity (Salahudeen et al.,

2000). Additional cell injury may be caused by increased storage time and lipid peroxidation of cell membranes (Nakao et al., 2005). Therefore, the maintenance of membrane integrity during ischaemia and reperfusion is one hallmark of good protection of organ-preservation solutions (Southard and Belzer, 1995). Interestingly, the addition of curcumin to Celsior solution resulted in preservation of cellular membrane integrity as well as cellular metabolism in cells exposed to cold storage and rewarming. These protective effects of curcumin are not mediated by the haem oxygenase system, since the employment of siRNA for HO-1 and SnPPIX did not inhibit the cytoprotective actions of curcumin, which indicates that the cytoprotective effects of curcumin are predominantly HO-1 independent. It is possible that other endogenous phase II enzymes are involved in the cytoprotective actions of curcumin, furthermore, curcumin-induced gene and protein expression of HO-1 is not epitomized at the enzymatic level due to the reduction in metabolic and enzymatic activity associated with cold temperature (McLaren and Friend, 2003). These findings support the strategy of adding antioxidants to preservation solutions to further boost their antioxidant capacity, and endorse the hypothesis that preconditioning of organs to oxidative stress before harvest might limit cold storage- associated organ injury.

It should be noted that the cell culture model used here might not be fully reflective of the actual cellular conditions in preserved whole organs, because cells in the cultured settings are not disrupted as they go into cold storage. Furthermore, this model is also unable to separate between cold storage and rewarming, because the assays used to assess cytotoxicity do not provide information to differentiate between necrotic cell death observed in cold storage

and apoptotic cell death cell that occur during rewarming. However, the model used here partially simulates the cold storage situation, and provides valuable information that can not be readily obtained from *in vivo*.

One of the most promising strategies that lengthen the time of cold ischaemia without risking reperfusion injury is preconditioning, in which the graft is subjected to a modifying agent for a limited period of time before harvesting (Tsuchihashi et al., 2003) (Redaelli et al., 2002a). This preconditioning confers resistance to subsequent lethal stress by inducing the expression of the HSPs, which protect the cellular machinery of many organs from a wide variety of insults such as anoxia, ischaemia, and oxidative stress (Calabrese et al., 2003), HSP 32, better known as HO-1 is one of the most important of these (Maines and Gibbs, 2005). Many of the protective effects of HSPs have been attributed to HO-1(Maines, 1997). The expression of HO-1 is amenable to pharmacologic manipulations (Hill-Kapturczak et al., 2001). Its pharmacologic induction attenuated oxidative injury in a rat kidney ischaemia-reperfusion model (Maines et al., 1999) and improved survival after transplantation of fatty livers in rats (Amersi et al., 1999).

Therefore, pharmacologic induction of HO-1 might be of clinical value to permit longer cold storage of harvested organs before transplantation, and is an attractive approach to minimize preservation injury in organs (Balogun et al., 2003a) (Shibahara et al., 2002). From the perspective of the present study, it would be of interest to determine whether preconditioning of Girardi cells with curcumin, an inducer of HO-1 would reduce cold storage. It is interesting to observe that under these conditions curcumin induced HO-1 mRNA expression, suggesting that curcumin is a potent inducer of HO-1 and that Girardi cells are

still responsive even after a prolonged cold storage period, under these conditions, curcumin up-regulated haem oxygenase activity, and no sign of toxicity was detected in cell viability assays. The addition of haemin in the protocol was based on the idea that it would synergize with curcumin to up-regulate HO-1 mRNA and at the same time, provide the substrate for haem oxygenase enzymatic activity. It is worth noting that the increase in activity detected under the programmed change in temperature was lower than that observed during normothermia, since cold temperature is associated with reduced metabolic and enzymatic activity (Southard and Belzer, 1995). The results obtained in Girardi cells are similar to those obtained in renal epithelial cells, where the experiments examining the effect of curcumin on HO-1 mRNA during hypothermia were performed (Balogun et al., 2003a).

The findings in the present study suggest that experimental strategies using a programmed change in temperature could be used to protect organs prior to transplantation.

# 8 GENERAL DISCUSSION

## 8.1 Analysis of Methodology

### 8.1.1 Cell Culture Methodology

In this thesis, established commercial cell lines were used instead of primary cell lines because of their convenience. Primary cultures are of mixed nature, with limited culture lifespan and potential contamination problems. In contrast, the cell lines are more stable and less labour intensive to maintain. Admittedly, the advantages of primary cultures are that the cells have not been modified in any way (other than by enzymatic or physical dissociation). The following cell lines were used: Bovine Aortic Endothelial Cells (BAEC), RAW 264.7murine macrophages, and human cardiac myoblasts (Girardi cells). In this thesis, three *in vitro* models of cardiovascular tissue dysfunction were used; a model of $H_2O_2$-mediated by oxidative stress, a model of inflammation, and a model of hypothermic ischaemic injury. These models were used to study the major aspects of the pathophysiology of cardiovascular dysfunction, and to study the potential cytoprotective effects of HO-1 up-regulation in these models. In the present study, the three major cellular components which are involved in the pathogenesis of cardiovascular diseases were studied, i.e. endothelial cells (represented by BAEC), macrophages, which are involved in the pathogenesis of the inflammatory response associated with cardiovascular dysfunction (Schiffrin, 2002), and cardiac cells. BAEC were used before to establish the effect of 2-HC on haem oxygenase activity and HO-1 expression (Foresti et al., 2005), in addition to studying the potential anti-oxidant effects of 2-HC; since the endothelium plays a major role in tissue response to oxidative stress (Singh

et al., 2005). RAW 264.7 macrophages are established cell line and they have been extensively used to study the inflammatory response (Alcaraz et al., 2004). An LPS-mediated inflammatory response in RAW 264.7 macrophages was used because it is an established *in vitro* model of inflammation (Vicente et al., 2001). In the present study, this model was used to explore the different aspects of the anti-inflammatory response and the role of HO-1 in and CO was also elucidated. It is important to note that inflammation is considered as a major player in the patho-physiology of cardiovascular disease (Schiffrin, 2002). Therefore, we examined the effect of 2-HC on LPS-elicited inflammatory response, in addition, the role of HO-1 in this action was also examined. Furthermore, we used CO-RMs, to study the effect of CO, one of the products of HO-1 pathway, on the inflammatory response elicited by LPS.

Girardi cells (human atrial myoblasts) are established cell line (Harwood et al., 2003). In the present study, Girardi cells were used to establish the effect of curcumin on haem oxygenase activity and HO-1 expression, and we also used them to examine the potential cytoprotective effects of curcumin against oxidative and hypothermic damage on cardiac cells. Data obtained using Girardi cells is supported by a number of reports, for example, the response of these cells to $H_2O_2$ was documented in other studies (McDonald et al., 2000). However, so far, no data in the literature was provided about the expression of HO-1 in these cells. Data presented in this thesis provided a novel insight to the biological properties of Girardi cells, and therefore facilitated further studies to understand the patho-physiology of cardiac cells.

### 8.1.2 Biochemical Assays

#### 8.1.2.1 Haem Oxygenase Assay

The haem oxygenase activity assay was first described by Tenhunen *et. al.* in 1968 and has been used extensively by our laboratory to measure intracellular and tissue haem oxygenase activity (Foresti et al., 1997). The assay used in this thesis reflects the relative haem oxygenase activity in the sample as "pmol. bilirubin/mg prot./h". Although the value obtained is not directly comparable to the amount of bilirubin measured in the culture medium after stimulation of the HO-1 pathway, the fact that the substrate (haemin) and the cofactors NADPH, glucose-6-phosphate, glucose-6-phosphate-1-dehydrogenase and biliverdin reductase were all added in excess and the procedure was carried out in controlled conditions must be considered when interpreting the data.

### 8.1.3 Molecular Biology Techniques

#### 8.1.3.1 Western Blot Assay

Western Blot is an established molecular biology technique, which is used for measuring relative protein expression in cells and tissue, therefore every assay was run with both negative and positive controls (untreated cells/tissue and recombinant HO-1 for protein respectively) and β-Actin for equal loading. Since Western Blot analysis of HO-1 expression is only used in conjunction with haem oxygenase activity in this study, the actual levels (μg, μmoles etc) of HO-1 protein are not necessary; each experiment is presented by a representative blot which has been scanned into a microcomputer, cropped to the right size and printed.

### 8.1.3.2 TNF-α and IL-10 Detection by ELISA

For the determination of TNF-α and IL-10 production in cells stimulated with LPS, commercially available ELISA kits were used because of their high sensitivity and specificity for detection of TNF-α and IL-10 in murine cells (Abuarqoub et al., 2005). Data obtained using these kits are similar to the data obtained by other studies in the literature (Sawle et al., 2005).

### 8.1.3.3 SiRNA

RNA interference is the biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by targeting mRNA, resulting in a reduction in the expression of a particular gene in mammalian cells (Shan et al., 2004). In the present study, siRNA specific for HO-1 were used to determine the involvement of HO-1 in various cytoprotective effects of 2-HC and curcumin. SnPPIX was also used to test the involvement of HO-1 in different processes, however, SnPPIX is not a specific inhibitor for haem oxygenase enzymatic activity, it also inhibits other haem containing enzymes (Serfass and Burstyn, 1998). So a more clean and specific approach was needed to verify the role of HO-1, therefore, siRNA technology was used in this thesis. In addition, siRNA for the PI3K pathway were also used to determine the involvement of this pathway in 2-HC-mediated induction of HO-1. Furthermore, in this thesis, commercially available inhibitor for the PI3K was also used, however, there are limitations to this inhibitor, including a possible interaction with the components of growth medium (FBS) (Martin et al., 2004). Therefore, we used siRNA for the PI3K pathway as a more specific approach.

### 8.1.4 Cell Viability Assays

To assess cell viability three methods were used in parallel, LDH release, Trypan Blue exclusion and Alamar Blue assay.

#### 8.1.4.1 LDH

LDH is a cytosolic enzyme present within all mammalian cells, the normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid (Luss et al., 2002). LDH release is suggested to be a reliable method of assessing cytotoxicity because *in vitro* release of LDH from cells provides a reliable method for measuring cell membrane integrity and cell viability (Salahudeen et al., 2000) (Venkatesan, 1998).

#### 8.1.4.2 Trypan Blue

Trypan Blue is a vital dye; all cells which exclude the dye are viable. It is considered as a reliable method of assessing cell death (Byler et al., 1994). It has been used in numerous studies to assess cell damage and in particular cell membrane integrity (Kuramochi et al., 2004) (Fukuta et al., 1984) (Pinsky et al., 1995).

#### 8.1.4.3 Alamar Blue

This dye presents numerous advantages over other cytotoxicity or proliferation tests. First, it is simple to use, it is added directly to cells in culture towards the end of the incubation period (Nakayama et al., 1997). No additional reagents or manipulations are required. Second, Alamar Blue is non-toxic to the cells and user (Ahmed et al., 1994). Third, the Alamar Blue assay does not require any special handling or disposal methods since no radioactive or toxic materials are

used (O'Brien et al., 2000). Thus, it is less costly than traditional assays. Each cell line has unique metabolic properties and must be individually characterized to determine the experimental parameters (e.g., incubation time, Alamar Blue dilution) for optimal conversion from the oxidized to reduced forms of Alamar Blue. Recently, the dye has gained popularity as a very simple and versatile way of measuring cell proliferation and cytotoxicity.

## 8.2 Thesis Discussion

HO-1 is an inducible stress protein, the expression of which can be markedly augmented in eukaryotes by a wide range of substances that cause a transient change in the cellular redox state (Motterlini et al., 2002b). Induction of HO-1 plays an important role in the patho-physiology of several diseases involving the heart (Motterlini et al., 1998). Studies aimed at the development of novel inducers of the endogenous HO-1 gene or targeted gene over-expression of HO-1 serve as a therapeutic and preventive modality in patho-physiologic states (Scapagnini et al., 2002). Therefore, strategies to target and achieve regulated expression of HO-1 will have significant therapeutic implications in several pathological conditions of the cardiovascular system (Agarwal and Nick, 2000). The hypothesis behind the work carried out in this thesis is: up-regulation of the haem oxygenase activity and HO-1 expression by plant-derived phytochemicals offers protection to cardiovascular tissue against oxidative and inflammatory damage.

In Chapter 3, we established that 2-HC is a potent inducer of haem oxygenase activity and HO-1 protein expression in endothelial cells, both in a time and a concentration-dependent manner. This induction occurs at the transcriptional level as actinomycin D completely eliminated 2-HC-mediated activation of haem oxygenase. Furthermore, the results related to the transcription factor Nrf2 suggest that the latter is a potential transcriptional target for 2-HC. Furthermore, our data showed that 2-HC was well tolerated by cells as tested by LDH release and Alamar Blue assays. Our results are in agreement with data in the literature, Alcaraz and co-workers demonstrated that a synthetic chalcone 3', 4', 5', 3, 4, 5-hexamethoxy-chalcone was a potent inducer of HO-1 and it resulted

in the activation of Nrf2 expression (Alcaraz et al., 2004). Furthermore, our group showed that 2-HC was a potent inducer of haem oxygenase activity and HO-1 protein expression in BAEC (Foresti et al., 2005). In addition, we demonstrated for the first time that 2-HC-mediated induction of haem oxygenase activity and HO-1 protein expression is mediated by the PI3K pathway. However, our results showed MAPK pathways play a minor role in the 2-HC-mediated induction of HO-1. Few studies have demonstrated the cellular signalling mechanisms that mediate the 2-HC-elicited HO-1induction (Alcaraz et al., 2004). However, several studies demonstrated the ability of chalcones to regulate MAPK-responsive pathways (Frigo et al., 2002). These results are in agreement with data obtained by our group in which the authors concluded that MAPK play a minor role in the 2HC-mediated HO-1 expression (Foresti et al., 2005). However, more studies are needed to elucidate the molecular and cellular targets of 2-HC. Our data also showed that 2-HC protected endothelial cells against $H_2O_2$-medated cytotoxicity. These findings support the data in the literature (Foresti et al., 2005) in which the potent cytoprotective properties of chalcone were demonstrated, mainly through its ability to strongly activate the endogenous cytoprotective pathways (Alcaraz et al., 2004). By virtue of its polyphenolic chemical structure (Batt et al., 1993) (Dinkova-Kostova et al., 2001) 2-HC possesses antioxidant and free radical-scavenging characteristics (Herencia et al., 2002). Having established that 2-HC is a potent inducer of haem oxygenase activity and HO-1 protein expression; 2-HC was used in chapter 4 *in vitro* model of inflammation. Inflammation plays an important role in cardiovascular diseases (Davi and Falco, 2005). The present study demonstrated that 2-HC is an anti-inflammatory compound that inhibited the

inflammatory response in LPS-macrophages model. 2-HC reduced the LPS-mediated increase in nitrite, TNF-α production and iNOS expression; these effects were mediated by HO-1. Therefore, 2-HC can be used as a potential therapeutic agent to mitigate cardiovascular pathologies which are mediated by oxidative and inflammatory stress. Our data is in agreement with data in the literature, in which 2-HC and other derivatives showed potent anti-inflammatory (Ban et al., 2004) (Batt et al., 1993) and anti-oxidant properties (Nakamura et al., 2003). Results in chapter 3 and 4 emphasized this data, and provided a mechanistic insight to the cytoprotective properties of 2-HC, which mainly involve the intrinsic ability of this compound to activate the endogenous cytoprotective enzymes, i.e. HO-1. Furthermore, this study identified some of the intracellular signaling pathways involved in the 2-HC-mediated induction of HO-1, in particular PI3K.

In chapter 5, we investigated the role CO, one of HO-1 pathway end products, in combating the inflammatory response elicited by LPS. For this purpose we used CO-RMs as a method of delivering CO. In chapter 5, we described a series of experiments designed to test the ability of novel water soluble CO-RMs to release biologically active CO. The CO-RMs tested were able to release CO in a time-dependent and controlled manner. In addition, the two new water soluble CO-RMs (CORM-43 and CORM-319) were investigated in an *in vitro* model of inflammation, using LPS-induced inflammation in RAW 264.7 macrophages. Our results showed that CORM-43 and CORM-319 exhibited inhibitory effects on the LPS-induced iNOS expression, nitrite and TNF-α production. Furthermore, treatment with iCO-RMs, the inactive form of CO-RMs that does not release any CO as confirmed by the myoglobin assay, did not

affect the nitrite or TNF-α production and iNOS expression, which further emphasizes our conclusion that the anti-inflammatory effects of CORM-43 and CORM-319 are not caused by the metal carbonyl, but are due to CO. Furthermore, our findings showed that CO released from CORM-43 and CORM-319 ameliorated inflammation in LPS-induced model of inflammation. Our results emphasized data obtained in the literature, which demonstrated the ability of CO gas to suppress pro-inflammatory responses in macrophage activation (Otterbein et al., 2000) (Otterbein, 2002). Furthermore, our group recently provided evidence that CO released from CO-RMs attenuated the LPS-mediated inflammatory response (Sawle et al., 2005). However, data in chapter 5 provide a mechanistic insight into the anti-inflammatory properties of CO-RMs; in this study, we investigated the cellular and molecular targets of CORM-43, our results showed that this compound targets the AKT pathway. We also studied the effect of CORM-43 on the LPS-mediated activation of NF-KB, our data was inconclusive, one cannot conclude the effects on NF-KB without undergoing a more specific method for determining the effect on NF-KB, for examples, EMSA scan (Ranjan et al., 2004) (Alcaraz et al., 2004).

In chapter 6, we aimed at establishing that curcumin, a polyphenolic compound which has intrinsic cytoprotective properties (Scapagnini et al., 2002) (Balasubramanyam et al., 2003) (Balogun et al., 2003b) (Ghoneim et al., 2002), was able to induce haem oxygenase activity and HO-1 protein expression in cardiac myoblasts (Girardi cells). In addition, we determined the cytotoxicity profile of curcumin in Girardi cells. Furthermore, we demonstrated that curcumin can be used as a preconditioning agent to protect cardiac myoblasts against oxidative (explored in chapter 6) and hypothermic (chapter 7) stresses. Our

data showed that curcumin in the range of (5-15 μM) was not toxic to Girardi cells, and it markedly up-regulated haem oxygenase activity and HO-1 expression. Therefore, in chapter 6 and 7, curcumin at 15 μM was used as a preconditioning agent. Our data showed that curcumin exhibited antioxidant activities and protected Girardi cells against $H_2O_2$-mediated oxidative stress. Preconditioning of cardiac myoblasts with curcumin resulted in enhanced resistance to $H_2O_2$-mediated oxidative damage; this effect was mediated by HO-1, since the inhibitor of haem oxygenase activity (SnPPIX) markedly reduced cytoprotection by curcumin treatment. It is tempting to conclude that the antioxidant properties of curcumin are in essence related to its ability to stimulate HO-1. However, it is possible that the protective effects of curcumin against the $H_2O_2$-induced cytotoxicity also result from its antioxidant and free radical scavenging properties (Patro et al., 2002); since pre-treatment with curcumin (removal of the medium which contains curcumin) was unable to protect Girardi cells from $H_2O_2$-induced oxidative damage. Our results are in line with data in the literature, (Cohly et al., 1998) (Balogun et al., 2003b). Having established the anti-oxidant effects of curcumin and its ability to induce haem oxygenase activity and HO-1 in Girardi cells, curcumin was used as a preconditioning agent in chapter 7; in which we examined the ability of curcumin to protect Girardi cells against prolonged cold preservation. Using a cell culture model of hypothermia and reperfusion, we investigated whether the addition of curcumin to Celsior solution would ameliorate the cold-associated injury in cardiac myoblasts and therefore, allow longer cold preservation of these cells. Our study demonstrated that cold storage of Girardi cells causes a marked increase in cytotoxicity primarily due to loss of cell membrane integrity. The

addition of curcumin to Celsior solution resulted in preservation of cellular membrane integrity as well as cellular metabolism in cells exposed to cold storage and rewarming. Using HO-1 siRNA, we found that the protective effects of curcumin were partially mediated by HO-1. up-to date, only two studies demonstrated the protective role of curcumin on hypothermic-damage (Balogun et al., 2003a) (Fuller et al., 2003). It is possible that the cytoprotective properties of curcumin are related to the intrinsic ability of curcumin to scavenge oxygen free radicals (Balasubramanyam et al., 2003). Similar data was obtained using inducers of HO-1 (Redaelli et al., 2002a) (Akamatsu et al., 2004) (Tsuchihashi et al., 2003) or the end products of the HO-1 pathway (Amersi et al., 2002). However, this study provided a novel approach to improving preservation of cardiac cells by using naturally occurring compounds. We suggest that using naturally-occurring compounds which have the intrinsic ability to enhance the activity of endogenous protective pathways can be used as potential therapeutic intervention (Balogun et al., 2003a). However, more studies are needed to clarify the mechanisms that mediate the protective effects of curcumin, possible mechanisms include: the induction of other endogenous cytoprotective enzymes and other cellular pathways, such as, MAPK, PI3K. Furthermore, this study did not provide data about the cellular events that mediate cell death during hypothermia, for example, the role of ROS and apoptosis, which have been associated with cold-induced cell death (Bartels-Stringer et al., 2003), (Burns et al., 1998). Numerous studies have highlighted the role of mitochondria in the cold-induced injury (Kuznetsov et al., 2004). However, the role of mitochondria in the cold-induced cell injury was not studied. Furthermore, this

study only tested one aspect of cell damage, i.e., cell membrane damage; however, more studies to test the role of adhesion molecules.

## 8.3 Conclusion

Up-regulation of HO-1 system offers promising new approaches to experimental work in cell biology. The use of pharmacological and genetic interventions for up-regulation of HO-1 in the managements of cardiovascular diseases appears to be especially promising. These pharmacologic strategies to regulate the HO-1 system could open up new therapeutic approaches for the effective management for a number of clinical disorders. This thesis has described the potential of 2-HC to activate the haem oxygenase pathway, explored and identified the possible molecular mechanisms involved for this response. Furthermore, in this study we have identified 2-HC as a potent anti-inflammatory and anti-oxidant compound. In addition, this study identified curcumin as a preconditioning agent, which can be used to protect cardiac cells against oxidative stress and hypothermia induced injury. And finally, we identified two novel CO-RMs (CORM-43 and CORM-319) and established the anti-inflammatory effects of these compounds; and explored the potential molecular mechanisms that mediate the anti-inflammatory effects of CORM-43 and CORM-319. Collectively, these findings reinforce the hypothesis that the haem oxygenase pathway and its products can be used as an effective method to protect cardiovascular tissues against oxidative and inflammatory stresses. And therefore, this approach can be used to counteract cardiovascular disease.

## 8.4 Future Perspectives

- Utilising a more advanced approach to cell culture, for example, a model of co-culture could be utilised, such as co-culture of endothelial cells and Girardi cells, and a co-culture model of inflammation using macrophages and endothelial cells.
- To utilize primary cells culture models and human cell lines to establish the effects of 2-HC and curcumin in these systems.
- To further elucidate the molecular and intracellular mechanisms of 2-HC-mediated HO-1 expression, in particular, the PI3K/ AKT pathways.
- To further explore and identify the molecular mechanisms and cellular signaling pathways that mediate the cytoprotective effects of 2-HC and curcumin.
- To further study CORM-43 and CORM-319 in different cells lines, such as endothelial cells, and identify the molecular and intracellular targets of these CO-RMs, in addition to further explore the interaction between CORM-43 and CORM-319 and the AKT pathway.
- To utilize CORM-319 (as a slow releaser of CO) in models of co-culture, and utilizing these models to study the interaction of CO-RMs with macrophages and endothelial cells.
- In vivo studies could be utilized to test curcumin and 2-HC to establish the potential protective actions of these compounds in models of inflammation and cold ischaemia-reperfusion injury.

HO-1 has grown an important position as a crucial endogenous enzyme as a result of the myriad of biological functions. There has been increasing evidence that phytochemicals induce a wide range of endogenous detoxifying enzymes including HO-1 However, more studies should be undertaken to further explore the regulatory mechanisms of HO-1 by phytochemicals, so as to understand the physiological function of the HO-1 system in the pathogenesis of vascular disease.

# PUBLICATIONS AND ABSTRACTS

1) **Hadil Abuarqoub**, Roberta Foresti, Colin J. Green and Roberto Motterlini. "Haem Oxygenase-1 Mediates the Anti-Inflammatory Actions of 2'-Hydroxychalcone in RAW 264.7 Murine Macrophages". American Journal of Physiology, Cell Physiology, November 2005.

2) Hadil Abuarqoub, Roberta Foresti, Colin J. Green and Roberto Motterlini. "Curcumin Protects Cardiac Myoblasts Against Cellular Damage During Cold Preservation". In preparation.

3) **Hadil Abuarqoub**, Roberta Foresti, Colin J. Green and Roberto Motterlini. "Haem Oxygenase-1 Mediates The Anti-Inflammatory Actions Of 2'-Hydroxychalcone In RAW 264.7 Murine Macrophages". Abstract presented in the Annual Winter Meeting of the Pathological Society of Great Britain and Ireland, January 2006, Cambridge.

4) Philip Sawle, **Hadil Abuarqoub**, Roberta Foresti, Colin J. Green and Roberto Motterlini. "The Anti-Inflammatory Effects of the HO-1/Carbon Monoxide Pathway in RAW 264.7 Murine Macrophages". Abstract presented at the annual Heme Oxygenase Conference, October 2005, Boston, USA.

5) **Hadil Abuarqoub**, Roberta Foresti, Colin J. Green and Roberto Motterlini. "Curcumin Protects Cardiac Myoblasts Against Cellular Damage during Cold Preservation". Society of Low Temperature Biology 41$^{st}$ Meeting, Abstract Book, page 22, September, 2005.

6) **Hadil Abuarqoub**, Roberta Foresti, Colin J. Green and Roberto Motterlini. "The Modulation of Haem Oxygenase Expression by Curcumin under Hypothermia". Society of Low Temperature Biology 41$^{st}$ Meeting, Abstract Book, page 25 September, 2004.

# MEETINGS

1) The 189th Meeting of the Pathological Society of Great Britain and Ireland, January 4-6, 2006, Robinson College, Cambridge, UK. (Poster Presentation). Abstract Book, page 24.
2) Heme Oxygenase Conference, October 6-9, 2005, Boston Park Plaza Hotel, Boston, MA, USA. (Poster Presentation).
3) Society of Low Temperature Biology 41st Meeting, September 14-16, 2005, University of York, York, UK. (Oral Presentation).
4) Society of Low Temperature Biology 40th Meeting, September 9-10, 2004, Royal Free Hospital, London, UK. (Oral Presentation).

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